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The effect of diet macronutrient content, infection and injury stress on larval and adult life-history traits in *Drosophila melanogaster*

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Abstract:

Dietary restriction (DR), the limitation of calories or a particular nutrient intake without malnutrition, extends lifespan and delays ageing across a range of taxa. To understand this response better and therefore its importance in the ageing process, it is important to understand the evolutionary basis of this response and its generality across environments. Several evolutionary hypotheses about why DR increases lifespan have been proposed, and one in particular suggests DR individuals are frailer and only live longer than non-DR individuals under benign laboratory conditions. Dietary macronutrients have been found to alter infection outcomes, potentially due to altered immune responses and changes in the rate of clearing of pathogen (resistance), or other effects of diet on host-parasite interactions including the ability to withstand a given pathogen load (disease tolerance). Individuals in other host-pathogen systems have been found to alter their diet choice as a result of infection. Adult DR responses and response to infection may also be altered by juvenile environmental conditions, as juvenile diets have been shown to have important effects for multiple adult life-history traits.

To understand the interactions between DR, injury and infection stress, and juvenile and adult environments, here I ask the following questions using the *Drosophila melanogaster* - *Pseudomonas entomophila* host-pathogen system, and diets differing in the ratio of macronutrients (protein to carbohydrate ratios, P:C): (i) Do additional stresses of injury and infection remove the lifespan benefit of DR, and are some diets better for *D. melanogaster* survival post-infection with *Pseudomonas entomophila?* (ii) Does larval dietary macronutrient manipulation affect adult life-history traits and survival post-infection? (iii) Do infected *D. melanogaster* individuals have altered diet preference post-infection with *P. entomophila*? and (iv) Does diet affect host resistance or disease tolerance with *P. entomophila* infection? By addressing these questions, I hope to improve our understanding of the evolutionary basis of the DR response and its generality across environments.

In chapter 2, I show that the benefits of DR response remain even with injury and infection stress, where with decreasing P:C, survival increases and the rate of ageing decreases, as does reproduction. Low P:C diets are particularly bad for survival post-infection with *P. entomophila*, and injury stress has no additional effect on survival in comparison to the control group. In chapter 3, I show that intermediate to high larval P:C increases measures of larval development and increases adult reproduction, however larval P:C does not alter adult lifespan or infection outcomes. In chapter 4, I show that short-term diet preference does not change with injury or *P. entomophila* infection. In chapter 5, I show that although higher P:C increases post-infection survival with *P. entomophila,* this may not due to increased resistance, as bacterial loads and a measure of the immune response to *P. entomophila*, AMP gene expression, are similar across two P:C diets. These data suggest a potential role of increased disease tolerance on the higher P:C diet with *P. entomophila*, requiring further study. Taken together, these data suggest that the response to DR, achieved through lowering P:C ratio, is relatively unaffected by the additional stresses of infection and injury; that adult, not juvenile, dietary macronutrient manipulation alters infection outcomes; and that this may be independent of changes in bacterial clearance. This suggests that the most likely evolutionary explanation for the DR response is that it is an adaptive shift in relative investment in life-history traits that is consistent across environments, particularly exposure to infection and injury. Furthermore, these results provide further evidence of differential effects of P:C depending on the host-pathogen pair, requiring further study to understand these complex interactions across systems.

Lay summary:

Diet has been on the forefront of multiple research areas due to its widespread effects on health. Changing different components of diet is complicated, as either how many calories are available can be changed, or only the overall quantities of nutrients, such as protein, carbohydrates or lipids can be changed. In experiments where diet is manipulated in different ways, one common result across different animal groups is that dietary restriction (DR), the limitation of the amount of food or of specific nutrients without malnutrition, increases lifespan, delays ageing, and decreases reproduction. This increase in lifespan with DR has been well-studied in a wide variety of animals from yeast to mice, and its generality suggests that it may be used to help with an ageing human population. However, while this response is common, it does not always appear in all situations. In addition, although several evolutionary hypotheses for this response have been proposed and tested, it is still not understood why lifespan increases with DR. Therefore, it is important to investigate these hypotheses further and to test DR in different environments to understand how general this response is.

From the several evolutionary hypotheses, one suggests that DR individuals are frailer than fully fed individuals are. They are proposed to live longer with a reduced diet only in laboratory conditions, as they would be too frail to survive stressors in wild conditions. According to this hypothesis, if individuals are stressed with additional stressors commonly found in the wild, for example injury and infection, DR individuals would not live as long as they are not able to deal with any additional stressors. Changes in diet also affect how individuals are able to deal with and survive infection. This might be because diet affects how the individual is able to prevent, respond to, or repair the damage caused by the pathogen. Outside of this, diet may also affect how the pathogen grows inside the individual, as the pathogen can use the increased availability of food to grow quicker. Once infected, individuals often also eat less food, but choose diets that are associated with increased survival after being infected. The diet that an individual eats early in its life can also have large consequences for the individual, and may also affect how

individuals deal with infection in adulthood, however this has not been tested where only this early-life diet is altered.

In this thesis, I will apply dietary restriction by keeping the caloric value of diets the same, but changing how much protein or carbohydrate is in the different diets. I will use a common laboratory organism, the fruit fly (*Drosophila melanogaster*), and apply stressors of injury or infection with a bacterial pathogen (*Pseudomonas entomophila*), to ask several questions about how diet and infection or injury interact, and whether infected individuals choose a diet associated with higher survival. These questions include testing this evolutionary hypothesis prediction about whether DR responses occur in more stressful conditions.

I show that injury and infection stressors do not remove the increase in lifespan with DR, and therefore that DR responses are present even with additional stressors. I also show that some diets are worse for surviving *P. entomophila* infection, as flies on low protein to carbohydrate diets had very poor survival after infection. I then show that, while larval macronutrient changes in diet affects adult reproduction measures, larval diet does not affect adult survival after *P. entomophila* infection. I also show that infection with *P. entomophila* does not alter food choice, and that diet does not appear to change the growth of bacteria, or a measured immune response, suggesting instead that diet may be altering another aspect in the flies outside of changes in control of bacterial growth. These results suggest that DR in terms of changes in protein to carbohydrate content in diet can extend lifespan even with additional stressors of injury and infection, and that early-life diet does not affect this. In addition, with *P. entomophila* infection, flies do not choose diets that are more beneficial to them after infection, and changes in survival may not due to diet affecting changes in bacterial growth. Overall, this suggests that DR responses still appear in more stressful environments with injury and infection stressors, while changes in nutrients affects how an individual responds to infection with *P. entomophila* infection.

Declaration:

The work and experiments in this thesis are composed by myself, with guidance from my supervisors, unless explicitly stated below and in text. This work has not been submitted for any other degree or professional qualification. I use "we" throughout chapters 2 to 5, as these chapters are written as papers. Fergal M. Waldron and Katy M. Monteith created and maintained the fly population that I used in all the work included in this thesis, and wrote the entire section about the creation of the fly population included in Appendix A, which is included as part of the publication involved with Chapter 2.

Chapter 2 has been published in Evolution as "Testing evolutionary explanations for the lifespan benefit of dietary restriction in fruit flies *(Drosophila melanogaster)*" (Savola et al., 2021) by *Eevi Savola*, Clara Montgomery, FMW, KMM, Pedro Vale, and Craig Walling. The data and associated R script are published on the Dryad repository (Savola et al., 2020a). The version in this thesis differs in the methods section due to inclusion of common methodology in Chapter 1. Prior to publication, this paper was also published as a pre-print (Savola et al., 2020b). I designed the experiment, with help from PV and CW. I did the experimental work with help from CM for the early part of the experiment, where I completed the data collection for the full experiment. Jonathon Siva-Jothy helped with choosing the estimated diluted dose used in bacterial work (noted in methods, data not shown), and Megan A. Wallace tested the DGRP outcross for the presence of common fly viruses (noted in methods, data not shown). I analysed the data with help and guidance from CW, and wrote paper with comments from CW, PV, FMW, KMM, CM, and two anonymous reviewers.

Chapter 3 has been published as a pre-print on BioRxiv as "Larval diet affects adult reproduction but not survival regardless of injury and infection stress in Drosophila melanogaster" (Savola et al., 2020c) by *ES*, PV and CW. The version in this thesis differs in the methods section from the published version due to inclusion of common methodology in Chapter 1. I designed the experiment with help from PV and CW. I completed the data collection, with help at the end of the

experiment from KMM, Paul McNeil and Paulina Mika. Confirmation of infection from one day of missing data was provided by Anu Halonen (Appendix C, data not shown). I analysed the data with help from CW, and wrote the paper with comments from CW, and PV.

I designed the experiment in Chapter 4, with help from PV and CW. I completed the experiment, and analysed the data with guidance from CW, PV and Joshua Moatt. I wrote the chapter with comments from CW, PV and JM.

In Chapter 5, I designed the experiment with help from PV and CW. I completed the study, with KMM helping with the initial RNA and cDNA work involved in the antimicrobial peptide gene expression assay. I analysed the data with help from CW and PV, and wrote the paper with comments from CW and PV.

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Table of Contents:

Chapter 1:

General introduction

1.1 Dietary restriction (DR):

Trade-offs between different life-history traits are common, as an increase in fitness associated with allocation to one life-history trait is often associated with a decrease in allocation to another trait, which carries its own negative fitness effect(reviewed in Stearns, 1989; Shanley & Kirkwood, 2000). Life-history traits include traits such as survival, reproduction, growth, and development, such as size and age at maturity (reviewed in Stearns, 1976, 1989). A well-studied trade-off between these life-history traits is between lifespan and reproduction (reviewed in Stearns, 1989, 2000; Shanley & Kirkwood, 2000). Environment can have profound impacts on life-history trade-offs (reviewed in Stearns, 2000). One of the most consistent environmental manipulations to extend lifespan and reduce ageing, whilst also reducing reproduction, is dietary restriction (DR) (reviewed in Mair & Dillin, 2008; Fontana et al., 2010; Simpson et al., 2017).

DR is where either the overall calorie or a particular nutrient intake is reduced without malnutrition (reviewed in Speakman & Mitchell, 2011; Simpson et al., 2017). DR has been found to increase lifespan in a diverse range of taxa, for example *Saccharomyces cerevisiae* yeast (Jiang et al., 2000), *Caenorhabditis elegans* nematodes (Klass, 1977), *D. melanogaster* fruit flies (Mair et al., 2005; Lee et al., 2008), *Teleogryllus commodus* field crickets (Maklakov et al., 2008), *Gasterosteus aculeatus* stickleback fish (Moatt et al., 2019), Labrador Retriever dogs (Kealy et al., 2002), *Microcebus murinus* mouse lemurs (Pifferi et al., 2018) and *Macaca mulatta* rhesus macaques (Colman et al. 2009, but see Mattison et al. 2017; Pifferi and Aujard 2019 for discussion concerning differences in survival between studies). As the lifespan response to DR is found across taxa, DR research has received a lot of attention due to the potential use of DR, or DR mimetics, as ageing interventions in humans in an ageing human population (reviewed in Speakman & Mitchell, 2011; Le Couteur et al., 2016; Balasubramanian et al., 2017). In addition, it has allowed a better understanding about conserved mechanisms affecting ageing and lifespan across taxa and to understand variation in lifespan (reviewed in

Fontana et al., 2010; Gems & Partridge, 2012; Fontana & Partridge, 2015; Kapahi et al., 2017).

In this chapter, I will first explain the current knowledge about DR responses and known variation in this response, and outline the associated proposed evolutionary hypotheses for the DR lifespan extension response. Then, I will review how changes in early-life diet affects later life-history traits, and outline which diets individuals prefer when given a choice. Next, I will focus on how different diets affect infection outcomes. Briefly, I will discuss whether DR methods could be applicable in humans. After outlining the research aims for this thesis, I explain how a common laboratory organism, fruit fly *Drosophila melanogaster,* responds to infection, in particular to a bacterial pathogen of *Pseudomonas entomophila. F*inally I outline common methodology used in this thesis.

1.2 Variation in DR responses:

There is considerable evidence of variation in DR responses across studies. Understanding what causes variation in DR responses is important, as DR has been considered to be applied in humans to aid with an ageing society, and to slow appearance of old-age pathologies (see section 1.8). Here, I outline several lines of evidence indicating that DR responses can vary.

1.2.1 How does the type of diet affect DR responses - calories or nutrients?

Much of the early DR research focused on the reduction of calorie intake without malnutrition (for details on the history of DR research see e.g. Masoro 2005; Speakman and Mitchell 2011; Solon-Biet et al. 2015). Often cited as the first study to find a lifespan extension effect of reduced calorie intake was a study in 1935 conducted in rats (McCay et al. 1935, but see discussion in Speakman and Mitchell 2011; Speakman et al. 2016 for previous related studies prior to 1935). Other changes in diet also affect lifespan, for example the limitation of specific nutrients can lead to increased lifespan (reviewed in Simpson & Raubenheimer, 2009; Speakman & Mitchell, 2011). Accordingly, manipulation of various macronutrients including protein, carbohydrates and lipids across various taxa have

highlighted the importance of specific nutrients in DR responses (reviewed in Simpson & Raubenheimer, 2009; Speakman et al., 2016; Simpson et al., 2017). From a meta-analysis of a range of diet manipulations across taxa, there is evidence that protein restriction may account for larger changes in lifespan, however caloric restriction also affected lifespan, and this analysis included relatively few studies where protein intakes were altered (Nakagawa et al., 2012). It has also been suggested that calories account for more of the change in lifespan in rodent studies, or that the method of restriction (dilution vs. absolute quantity of food) may alter results (Speakman et al., 2016). Overall, even after extensive studies, the role of calories and nutrients and their interactions are still largely debated (reviewed in Masoro, 2005; Speakman et al., 2016; Simpson et al., 2017). Many studies only manipulate the amount of a particular nutrient without considering other aspects of the diet, including the availability of other nutrients or changes in calories, further making comparisons between calories and nutrient availability difficult (reviewed in Simpson et al., 2015).

A crucial approach that has highlighted patterns in various traits including lifespan between changes in calories and nutrients is the geometric framework of nutrition (GF, reviewed in Simpson and Raubenheimer 2012; Simpson et al. 2017). Here, often the quantities of two macro- or micronutrients are altered, so that diets range in both caloric and nutrient composition, where changes in a particular trait can then be plotted onto a nutrient space of dietary parameters (reviewed in Simpson et al., 2017). Such studies can therefore evaluate the relative importance of overall caloric value or protein to non-protein content of the diet in DR responses, by measuring traits such as lifespan and reproduction and accounting for individual intakes of particular diets (reviewed in Simpson et al., 2017). GF studies have the advantage of incorporating individual intakes, and therefore also account for changes in nutrient intake due to processes such as protein leverage, where more or less of a particular protein:non-protein diet is eaten to maintain protein intake at a given level (reviewed in Simpson et al., 2017). Individual level intakes are however not always included in all GF studies (Jang & Lee, 2018; Kutz et

al., 2019). Studies implementing GF approaches highlight that the relative amount of macronutrients, often protein to non-protein content, and not calories, are responsible for changes in lifespan in vertebrates and invertebrates (e.g. Solon-Biet et al. 2014; Le Couteur et al. 2016; Simpson et al. 2017; Moatt et al. 2019, but see review Speakman et al. 2016). Protein content may not be the sole reason for altered lifespan with only changes in P:C, as in many studies, carbohydrates also have a significant effect on lifespan (e.g. Maklakov et al., 2008; Solon-Biet et al., 2014; Jang & Lee, 2018; Moatt et al., 2019).

In insects, where the majority of GF studies have been completed, suggest that mainly protein to carbohydrate (P:C) ratios, and not calories, alter lifespan responses to diet (Lee et al., 2008; Maklakov et al., 2008; Jensen et al., 2015; Le Couteur et al., 2016). For example, female *D. melanogaster* fed 28 liquid diets of varying in P:C and concentration of macronutrients, individuals lived longest on a very low P:C ratio of 1:16 P:C, whereas lifetime reproduction peaked at a higher P:C ratio of 1:4 P:C (Lee et al., 2008). Caloric restriction did not explain changes in lifespan, as lower calories did not extend lifespan (Lee et al., 2008). Similar findings of low P:C diets extending lifespan have been found in other studies in *D. melanogaster* (Jensen et al., 2015), and other insect species (Maklakov et al., 2008; Fanson et al., 2009, 2012; Harrison et al., 2014), suggesting P:C is more important than calories in DR responses in insects (but see results of decreasing calories in Jang & Lee, 2018). While this pattern of increased lifespan on very low P:C is common, it does contrast with other insect studies finding lifespan is increased on intermediate P:C diets (Carey et al. 2008; Skorupa et al. 2008; Lee 2015; Jang and Lee 2018; Kim et al. 2020, but see results for males in Kim et al. 2020). Diet delivery in *D. melanogaster* may explain these differences in lifespan, as the studies in *D. melanogaster* finding that lifespan is maximised at very low P:C diets apply liquid diets, resulting in relatively lower lifespans on all diets (Lee et al., 2008; Jensen et al., 2015), which may alter observed patterns in lifespan. CAFE assays use capillaries filled with liquid diet, where the flies are required to eat upside down, which may contribute to these differences in comparison to eating

solid foods in laboratory conditions (reviewed in Deshpande et al., 2014; Marx, 2015). It should be noted that dietary protein:fat is important for insect carnivores (e.g. Jensen et al., 2012; Al Shareefi & Cotter, 2019), however arthropod and vertebrate carnivores seek out carbohydrate sources as well (reviewed in Simpson et al., 2014). In *D. melanogaster*, changes in lipids do not affect lifespan (Grandison et al., 2009) or larval development (Reis, 2016), and therefore are often kept constant, however further study using chemically defined diets where only lipids are altered should be considered to test this further also in other insects. Overall, changes in dietary P:C appear important in altering lifespan in insects, and therefore in this thesis, diets are altered as isocaloric P:C changes using an insect species *D. melanogaster* (see sections 1.10 and 1.11).

Although changes in P:C ratios affect lifespan, further studies manipulating micronutrients suggest DR responses may be due to more detailed changes in diet, for example from the availability or concentration of particular or types of amino acids (Orentreich et al., 1993; Grandison et al., 2009; Piper et al., 2017; Le Couteur et al., 2020). Diets are complex, and subsequently, there may be other micronutrient changes. For example, in *D. melanogaster,* changes in P:C are often manipulated in the ratio of yeast to sugar (e.g. Lee et al., 2008; Skorupa et al., 2008; Bruce et al., 2013). Yeast also contains other nutrients such as lipids and carbohydrates, and also includes other micronutrients (Simpson & Raubenheimer, 2009; Lee, 2015), including sterols such as cholesterol (Piper et al., 2014; Zanco et al., 2020). Altering cholesterol in diet affects lifespan responses by interacting with macronutrients, for example by increasing lifespan of flies with otherwise low lifespan on high P:C, potentially due to depleted sterol resources due to high egg production (Zanco et al., 2020). Chemically defined diets, where the exact sources of nutrients are finely controlled, have been developed and used in studies to confirm P:C ratio outside of other changes affects lifespan in insects (Jensen et al., 2015; Lee, 2015; Jang & Lee, 2018). In terms of amino acids, if diets are composed to match a species' exome levels of required amino acids from genome translation, these diets decreased overall food intake, and increased early-life reproduction

without reducing lifespan in *D. melanogaster* (Piper et al., 2017). In mice, exome matched diets increased growth (Piper et al., 2017). This suggests that diets can be constructed that can maximise both lifespan and reproduction due to a targeted, but a lower overall, amino acid content (Piper et al., 2017). While changes in micronutrients are important in understanding which components of diet are affecting DR responses, such detailed changes in diet are not investigated in this thesis. Instead, broader effects of isocaloric P:C are applied through yeast:sugar ratios with associated changes in micronutrients (see section 1.11.2).

1.2.2 Do males and females respond to DR differently?

From studies that use both male and females to measure effects of DR, sex differences in the response to DR have been reported in both vertebrates and invertebrates (Maklakov et al., 2008; Nakagawa et al., 2012; Jensen et al., 2015; Moatt et al., 2016, 2019). For example in insects, females show larger increases in lifespan with DR than males (Magwere et al., 2004), males and females have slightly different P:C optima for reproduction (Maklakov et al., 2008; Jensen et al., 2015), and females have higher requirements for micronutrients such as sterols and vitamins than males (Wu et al., 2020).

These differences have been suggested to be due to differences in costs of reproduction, where males do not experience large costs in reproduction in comparison to females, and therefore would not benefit from increased nutrition to the same degree as where costs are higher in females (reviewed in Bonduriansky et al., 2008). The differences may also be due to differences in life-history investment strategies, where females invest more resources into reproduction and may require more of a particular nutrient, such as protein for egg production (reviewed in Wheeler, 1996; Mirth et al., 2019). Males instead require more carbohydrates to fuel behaviours involved in acquiring mates or from competition (Maklakov et al., 2008; Jensen et al., 2015). However, differences in the apparent optima may also be due to how males in laboratory conditions are not exposed to important costs in relation to reproduction such as competition (Moatt et al., 2016). Finally, these changes in how males and female *D. melanogaster* respond to DR

have been suggested to be due to interactions of diet with gut epithelium integrity (Regan et al., 2016). DR in female flies slows down gut tears and abnormalities which develop with age, whereas male flies do not show large differences with age in gut integrity, and therefore DR does not have such a large effect on their lifespan (Regan et al., 2016). Altogether, DR responses appear greater when tested in females, potentially due to lack of appropriate costs of reproduction when tested in males (Moatt et al., 2016), and female measures of reproduction may be easier to measure due to direct measures, for example, of egg laying in insects. Therefore, in this thesis, due to this variation in DR responses between males and females, only female *D. melanogaster* will be used (see sections 1.9 to 1.11).

1.2.3 Does genetic variation affect DR responses?

There is considerable genetic variation in the response to DR. In *D. melanogaster*, when applying the same lower caloric diet to different genetic backgrounds, different lines have increased or decreased lifespan, or diet has no effect on lifespan (Jin et al., 2020; McCracken et al., 2020a). Similar patterns have been found in different mice strains (Liao et al., 2010; Rikke et al., 2010). One criticism is that often these studies only use two diets, and therefore it is possible that each genetic line might have the typical DR response given a range of diets, but shifted to a different restriction level (McCracken et al., 2020b). When applying multiple diet concentrations to several lines from the *Drosophila melanogaster* genetic reference panel (DGRP, Mackay et al., 2012), similar variation in DR responses was observed, suggesting that genetic variation in DR responses is present also when multiple diets are applied (McCracken et al., 2020b). In this thesis, an outbred DGRP population of *D. melanogaster* will be used, to ensure any measured patterns are not due to a single genotype response, and therefore should be more general (see section 1.11.1).

1.2.4 Does DR only extend lifespan in model species or in laboratory conditions?

As the majority of DR studies are completed using model species, it has been suggested DR responses may not be as apparent outside of such species and

by extension in the wild (Harper et al., 2006; Nakagawa et al., 2012). Laboratory animals differ to wild individuals in many ways, including selection for faster production of large numbers of offspring, and faster development and larger body size, leading to increased food intakes in laboratory individuals compared to wild individuals (reviewed in Harper et al., 2006). From two meta-analyses incorporating model and non-model system estimations, DR extends lifespan about twice as much in model species (Nakagawa et al., 2012) and reduces reproduction to a greater degree with increasing DR in model species (Moatt et al., 2016). However, in a non-model species of *G. aculeatus* stickleback fish, protein:lipid ratios and not caloric content affected lifespan, suggesting DR responses are present in non-model species (Moatt et al., 2019).

There is conflicting evidence about whether wild-caught individuals subjected to DR in the lab have increased lifespan with DR (Harper et al., 2006; Sutphin & Kaeberlein, 2008; Metaxakis & Partridge, 2013). In mice, grand-offspring of wild caught individuals did not show increased mean lifespan with DR, potentially due to wild caught individuals consuming less food under fully fed conditions (Harper et al., 2006; but see discussion in Mair & Dillin, 2008). In contrast, wild-caught *C. elegans* had increased lifespan in DR conditions through bacterial deprivation (Sutphin & Kaeberlein, 2008), similar to findings in *D. melanogaster* (Metaxakis & Partridge, 2013). The most applicable study to answer whether diet alters mortality in the wild offered protein or carbohydrate diets to wild *Protopiophila litigata* antler flies (Mautz et al., 2019). Protein diets increased mortality in both in laboratory and wild conditions, suggesting DR can affect mortality in wild conditions in a non-model species, however this effect was only apparent in one of the years tested (Mautz et al., 2019).

Overall, although there is evidence that DR extends lifespan also outside of laboratory studies, one additional way of testing whether DR would be applicable outside of benign laboratory conditions would be to subject laboratory individuals to additional stressors to measure whether lifespan is still extended with DR (Adler and Bonduriansky 2014, see sections 1.3 and 1.7). Therefore, in this thesis I will be

applying additional stressors of injury and infection to *D. melanogaster* in laboratory conditions to test whether DR responses are removed in less benign laboratory conditions (see section 1.11.3).

1.3 Evolutionary hypotheses of lifespan extension by DR:

The presence of lifespan extension with DR across a range of taxa suggests it is an evolutionarily conserved response acting through conserved mechanisms (reviewed in Fontana et al. 2010; Fontana and Partridge 2015). Consequently, understanding the evolutionary basis of the response is paramount in determining whether DR, or by extension DR mimetics, may be applicable in humans (see section 1.8). Despite much research, there is no consensus on the evolutionary mechanisms underpinning DR responses, and therefore testing of predictions from the various suggested evolutionary hypotheses are required (Zajitschek et al., 2016; Travers et al., 2020; Moatt et al., 2020).

There are four predominant evolutionary hypotheses which explain why DR extends lifespan: resource reallocation hypothesis (RRH, as in Regan et al. 2020) (Holliday, 1989; Shanley & Kirkwood, 2000), resource recycling hypothesis (NRH, as in Regan et al. 2020) (Adler & Bonduriansky, 2014), toxic protein hypothesis (as in Regan et al. 2020) (Fanson et al., 2009, 2012), and clean cupboards hypothesis (Speakman, 2020). In addition, a perspective shift of the current evolutionary and mechanistic processes underpinning DR and life-history trade-offs as a plastic response framework has been proposed, which considers diet as one of the important predictive cues of the environment, where multiple cues affect predictive plasticity and therefore increased fitness in the wild (Regan et al., 2020). This shift in perspective does not consider the aforementioned evolutionary theories as mutually exclusive, and suggests that the understanding of the evolution of DR responses requires considerations of DR from a more ecologically relevant context (Regan et al., 2020).

From the four DR evolutionary hypotheses, the toxic protein hypothesis suggests that with increasing protein, reproduction increases, however the toxic effects of protein metabolism reduce lifespan at higher protein intakes (Fanson et

al., 2009, 2012). The clean cupboards hypothesis suggests that under resource limitation, individuals are attempting to reach an "immediate energy balance" by utilising stored nutrients, such as fat stores, and through reduction in damaged organelles or other cellular processes (Speakman 2020, see also section 1.3.2 for similar descriptions of the mechanistic basis of the NRH). As a by-product, this leads to increased lifespan (Speakman, 2020). Here, I will focus on the two predominant evolutionary explanations in detail, as they make different predictions about whether DR will increase lifespan in natural conditions with additional stressors (Shanley & Kirkwood, 2000; Adler & Bonduriansky, 2014). It should be noted that both of these evolutionary hypotheses have been composed in terms of changes with caloric restriction, and one was originally formulated using laboratory rodent data (Shanley & Kirkwood, 2000; Kirkwood & Shanley, 2005). However, the predictions concerning whether DR responses are removed with additional stressors should still be valid in terms of changes in P:C (see review Moatt et al. 2020).

1.3.1 Resource Reallocation Hypothesis (RRH):

The most widely accepted hypotheses to explain the lifespan increase in response to DR is the Resource Reallocation Hypothesis (RRH) (Holliday, 1989; Shanley & Kirkwood, 2000). The RRH suggests that lifespan increases due to an adaptive reallocation of limited resources into somatic maintenance and away from reproduction (see Figure 1.1) (Shanley and Kirkwood 2000). This hypothesis is based on the disposable soma theory of ageing, which states that there is a trade-off between investing resources into reproduction and somatic maintenance (reviewed in Kirkwood and Holliday 1979; Holliday 1989). The RRH states that with unlimited resources, only a specific and constant amount of resources are allocated to somatic maintenance with the rest allocated into reproduction (Holliday, 1989; Shanley & Kirkwood, 2000). Reproduction itself also carries a cost to the individual (reviewed in Holliday, 1989; Shanley & Kirkwood, 2000).With reducing resources, an individual's fitness would not benefit from increased reproduction, as this would be costly to the parent both in terms of allocating already limited resources into

reproduction, and due to a likely low number of the offspring surviving (Holliday, 1989; Shanley & Kirkwood, 2000). Therefore, it would be more beneficial to temporarily invest resources away from reproduction and into somatic maintenance (Holliday, 1989; Shanley & Kirkwood, 2000). If individuals are kept on low food conditions permanently, but not in starving or malnutrition conditions, this consequently leads to reduced rates of ageing and longer lifespans (Shanley & Kirkwood, 2000). The RRH requires fluctuating environments, where fitness is increased for individuals when resources return to increased levels for allocation to reproduction (Shanley & Kirkwood, 2000; Adler & Bonduriansky, 2014).

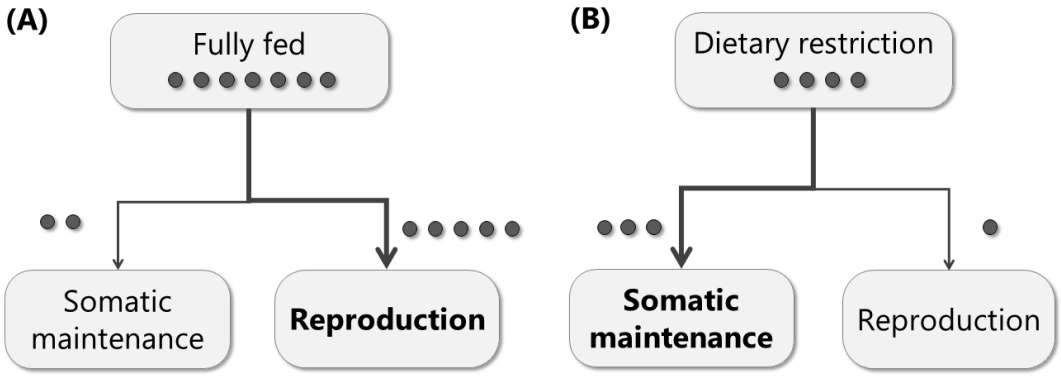

Figure 1.1: Summary of the resource reallocation hypothesis (Shanley & Kirkwood, 2000). Here resources are allocated between somatic maintenance (by extension survival), or reproduction. (A) In fully fed conditions, only a specific amount of resources are allocated to somatic maintenance and the rest into reproduction. This leads to increased reproduction, but lowered survival. Increased reproduction can reduce lifespan due to costs associated with reproduction. (B) With dietary restriction there are fewer resources. Most resources are allocated to somatic maintenance and away from reproduction, until better dietary conditions allow for more resources to be allocated to reproduction. This leads to higher survival due to increased somatic maintenance, but low reproduction. (Figure adapted from Adler and Bonduriansky 2014; Moatt et al. 2020.)

The RRH has received a lot of support due to a common finding where lifespan and reproduction are not maximised at a single diet, where instead female lifespan is often maximised at low P:C diets and reproduction at high P:C diets (e.g. Lee et al., 2008; Jensen et al., 2015; Moatt et al., 2019). However, this hypothesis has also received a wide-range of criticisms (Speakman & Mitchell, 2011; Regan et al., 2020; Moatt et al., 2020). The majority of criticisms of the RRH are related to the assumption that lifespan and reproduction trade-off with each other (see also review Speakman & Mitchell, 2011). For example, lifespan and reproduction can be decoupled in various ways in *D. melanogaster* and *C. elegans* (reviewed in Flatt, 2011).

There are many studies where no apparent trade-off between reproduction and lifespan is reported (O'Brien et al., 2008; Grandison et al., 2009; Jensen et al., 2015; Piper et al., 2017). For example, in male *D. melanogaster*, reproduction (as offspring production rate) and lifespan peaked at similar P:C ratios (Jensen et al., 2015), suggesting no trade-off as both traits can be maximised at one diet. In discussing studies such as this, it should be noted that trade-offs can appear without changes in allocation of resources (reviewed in Moatt et al., 2020). If lifespan and reproduction are not found to trade-off with each other with changes in dietary P:C, this may not indicate a trade-off does not exist, but rather that the two traits may peak around the same P:C ratio. Instead, these studies may indicate that the two traits do not differ in where they peak in the nutrient space, although they may be competing for the same resources at this P:C ratio (see further discussion in Moatt et al., 2020). Similarly, lifespan and reproduction can be maintained with exome matched diets and diet manipulation of amino acid availability can increase early-life reproduction without reducing lifespan (Grandison et al., 2009; Piper et al., 2017), however this can also be evidence that these diets did not remove the trade-off but the diets were better for maximising the two traits. When investment of resources into somatic tissues and reproduction was measured using isotype labelling of carbon and nitrogen of yeast, fully fed *D. melanogaster* invested more resources into both somatic maintenance and

reproduction than dietary restricted individuals, taken to suggest DR individuals do not invest more resources into somatic maintenance (O'Brien et al., 2008). However, some of the results from this study are comparable with the RRH, as DR individuals invested relatively higher amounts of resources into somatic maintenance (O'Brien et al., 2008), and again fully fed conditions may have been more optimal for somatic maintenance and reproduction. Finally, changing potential reproductive conditions by suppressing reproduction had no effect on DR lifespan responses in *D. melanogaster*, used to suggest a decoupling of the trade-off (Mair et al., 2004).

There are also criticisms not focusing on the trade-off itself. A study switching *D. melanogaster* from low to high P:C diets hypothesised that according to the RRH, individuals returning to fully fed conditions after restriction (from low to high P:C) should outcompete individuals kept in fully fed conditions (McCracken et al., 2020a). Against their prediction, individuals returning to fully fed conditions had increased mortality and lower reproduction in comparison to individuals kept in fully fed conditions (McCracken et al., 2020a). This could however indicate a cost of switching between diets instead of a cost associated with returning to fully feeding from DR conditions. Overall, this variation in lifespan and reproduction trade-offs together with other criticisms suggest that the RRH may not fully explain responses to DR. One major criticism of the RRH is that in the wild animals would not benefit from an increase in somatic maintenance as sources of mortality are more extrinsic, for example due to disease or predation, and therefore individuals would not benefit from delaying reproduction due to not surviving until favourable conditions return (Adler and Bonduriansky 2014, (see section 1.3.2).

1.3.2 Nutrient Recycling Hypothesis (NRH):

Another more recent evolutionary hypothesis, the NRH suggests that selection for any possible reproduction at lower food availability explains the DR response, and the lifespan extension response is a by-product of increased nutrient recycling mechanisms (Adler & Bonduriansky, 2014). The majority of studies in DR are conducted in benign laboratory environments, which are unlike more stressful

conditions in nature (Adler & Bonduriansky, 2014; but see Mautz et al., 2019). To fully understand whether DR, or DR mimetics, will benefit humans (see also section 1.8), we must first understand whether the DR response is consistent outside of a benign laboratory environment with additional stressors, such as ones found in natural conditions (Adler & Bonduriansky, 2014).

In brief, the hypothesis states that with lower nutrient availability and therefore lower nutrient-sensing pathway activation, nutrient recycling mechanisms such as apoptosis and autophagy are disinhibited (see Figure 1.2 and section 1.4) (Longo & Fontana, 2010; Adler & Bonduriansky, 2014). These upregulated processes together are proposed to use stored nutrients more efficiently and to use the available nutrients with a possible lower threshold for reproduction (Adler and Bonduriansky 2014). The increased nutrient recycling mechanisms also decrease the likelihood of developing old-age pathologies including cancers, which are common sources of mortality in the laboratory (Longo & Fontana, 2010; Adler & Bonduriansky, 2014). However, these mechanisms may also make the individual frailer and less able to respond to additional stressors such as infection or injury (Adler and Bonduriansky 2014). The NRH suggests that laboratory animals tested under benign conditions would have increased survival, however any additional stressors such as injury and infection would remove this lifespan benefit (Adler and Bonduriansky 2014). Therefore, the increase in lifespan with DR may only be a laboratory artefact, as individuals in the wild would face additional stressors and extrinsic mortality due to predation and infection, and wild individuals would likely not benefit from a decrease in old age-pathologies such as cancers that DR would protect against with old age (Adler & Bonduriansky, 2014).

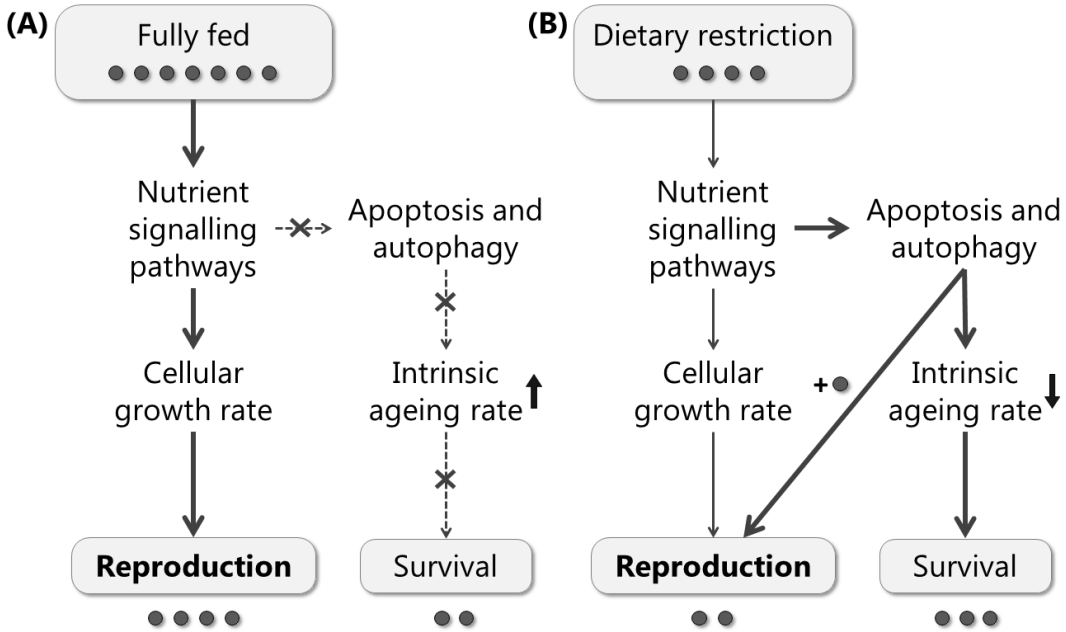

Figure 1.2: Summary of the nutrient recycling hypothesis (Adler & Bonduriansky, 2014). (A) Under fully fed conditions, nutrients activate the nutrient sensing pathways (IIS/TOR signalling), which increases cellular growth rate, and by extension reproduction and potential ability to deal with additional stressors. As cellular recycling mechanisms including apoptosis and autophagy are inhibited, the rate of intrinsic ageing is increased and lifespan is reduced. (B) With DR, nutrient sensing pathways are not as active and apoptosis and autophagy are disinhibited, leading to a lower rate of intrinsic ageing due to cell recycling mechanisms, and increased lifespan due to protecting against common laboratory causes of mortality, such as cancers. The cell recycling mechanisms allow the individual to use the available resources more efficiently, with a potential lower threshold for reproduction and possible further available resources for reproduction and lifespan. A reduced cellular growth rate due to lower activation of nutrient sensing pathways and increased apoptosis and autophagy may make the individual frailer to any potential additional stressors, and therefore lifespan should not be increased with DR with additional stressors. (Figure adapted from Adler and Bonduriansky 2014; Moatt et al. 2020.)

The NRH has received a considerable amount of criticism (Regan et al., 2020; see Moatt et al., 2020). Some of the predictions of the NRH have been tested, where predictions of the NRH have not received support. One of the key predictions of the NRH is that DR will not extend lifespan in the wild (Adler & Bonduriansky, 2014). In contrast, in wild *P. litigate* antler flies, protein supplementation increased mortality in one of the years tested, however diet had no effect on mortality in the other year (Mautz et al., 2019), suggesting the need for further study. An experimental evolution study in *D. melanogaster* hypothesised that according to the NRH, selection lines maintained under long-term DR, male flies should have higher reproduction and lifespan with DR due to increased efficiency at using the available resources (Zajitschek et al., 2016). Instead, only reproduction increased under DR conditions but there was no effect of selection diet on lifespan (Zajitschek et al., 2016). Finally, a study in *C. elegans* predicted that according to the NRH, inhibiting autophagy under DR conditions should decrease reproduction, however instead they found reproduction increased (Travers et al., 2020). However, one of the main predictions of the hypothesis has not been tested, where the NRH predicts that DR will not increase lifespan if stressors such as injury and infection are included in otherwise benign laboratory conditions (Adler & Bonduriansky, 2014).

1.3.3 Predictions of DR lifespan extension with additional stressors:

The two evolutionary hypotheses discussed here make very different predictions based on the different environments of laboratory or more natural conditions. The NRH states that the lifespan extension of DR would disappear with additional stressors, however the RRH does not make additional predictions based on the environment (see Figure 1.3 for potential survival results in experiments using additional stressors according to the predictions for the two hypotheses). Understanding whether additional stressors such as infection and injury, which are pervasive to human populations, remove the lifespan benefit of DR when measured in the laboratory is important to assess whether DR methods or mimetics may be suitable in ageing human populations (see section 1.8). In this thesis, I will be

addressing this question, by including injury and infection stressors to dietary restricted *D. melanogaster* to determine whether DR responses remain with additional stressors (see sections 1.9 to 1.11).

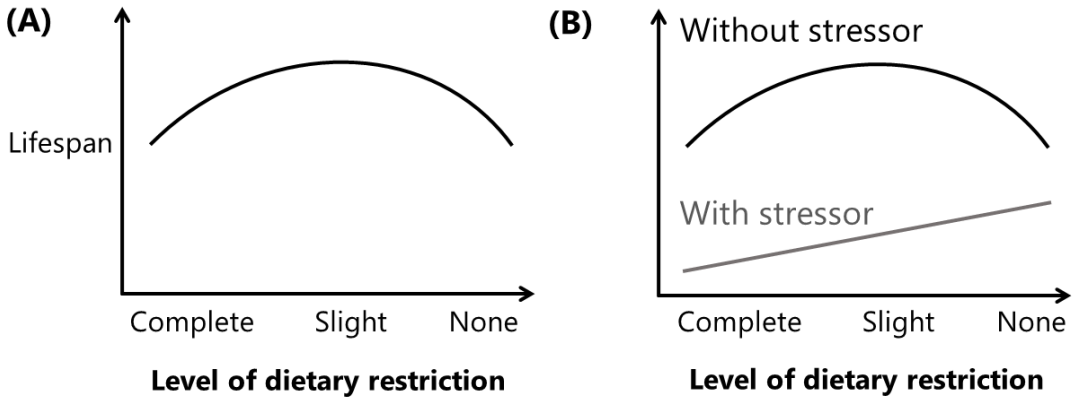

Figure 1.3: Predictions of effect of diet on risk ratios according to (A) resource reallocation (RRH) (Shanley & Kirkwood, 2000), or (B) nutrient recycling hypotheses (NRH) (Adler & Bonduriansky, 2014). (A) Under RRH, all stress treatments would have the same response to DR, where with DR lifespan increases up to a point, where with additional restriction, lifespan reduces again due to malnutrition. (B) Under NRH, only unstressed control individuals (black line) would have this typical DR response, and any additional stressors would remove the increase in lifespan with DR and individuals would have higher overall risk ratios (grey line). (Panel (A) adapted from Mair and Dillin 2008).

1.4 What is the physiological mechanism of DR?

Although well-studied, and included in evolutionary hypotheses explaining the lifespan extension of DR (see section 1.3), the mechanistic basis of the DR response is not fully understood (reviewed in Mair & Dillin, 2008). Several important genes and pathways involved in the DR response, which are found across taxa, are often part of nutrient signalling pathways, namely the insulin/insulin like-growth factor signalling (IIS) or mechanistic target of rapamycin (mTOR) pathways (reviewed in Mair & Dillin, 2008; Partridge et al., 2011; Regan et al., 2020; Pignatti et al., 2020). Altering the activity of the pathways affects lifespan in various taxa, for example lifespan increases when the activity of both pathways is inhibited (reviewed in Fontana et al., 2010; Pignatti et al., 2020) or when individuals are fed rapamycin, which targets the mTOR pathway, when tested in *D. melanogaster* or mice (reviewed in Selman, 2014). The IIS/mTOR nutrient signalling pathways respond to overall energy levels and types of nutrients such as amino acids, and regulate many downstream processes such as growth, metabolism, autophagy and apoptosis (reviewed in Regan et al., 2020). They also cross-regulate and are considered a network instead of separate pathways, together with other pathways linked to ageing such as sirtuin signalling proteins (reviewed in Pan & Finkel, 2017; Pignatti et al., 2020). The IIS pathway responds to insulin in vertebrates, or insulin-like ligands in other organisms and regulates processes such as cell growth and protein processing (reviewed in Regan et al., 2020). The mTOR pathways responds to amino acids and other nutrients and regulates processes such as cellular growth and protein synthesis (reviewed in Kapahi et al., 2010; Regan et al., 2020; Pignatti et al., 2020).

Under DR, these signalling pathways are less active (reviewed in Fontana & Partridge, 2015), and manipulation of these pathways can alter responses to DR (reviewed in Partridge et al., 2011). Under fully fed conditions, these pathways increase processes such as growth and synthesis of lipids, and with dietary restriction, growth is inhibited and nutrients are recycled (reviewed in Regan et al., 2020). Macro- and micronutrients can also affect these nutrient sensing pathways

(reviewed in Fontana & Partridge, 2015), suggesting P:C changes may affect lifespan through complex interactions with the IIS/TOR signalling pathways (reviewed in Simpson & Raubenheimer, 2009; Simpson et al., 2017). It should be noted that the IIS/mTOR pathways also respond to other cues outside of diet, including photoperiod, temperature, circadian rhythms, and infection (reviewed in Regan et al., 2020, for reviews for infection and diet interactions see also Ponton et al. 2013; Galenza and Foley 2019). Findings around the physiological mechanisms of DR have allowed for extensions of DR applications outside of changes in diets, by the production of many different DR mimetics focusing on these pathways, which may be more applicable in human populations (see section 1.8) (reviewed in Fontana et al., 2010; Speakman & Mitchell, 2011; Selman, 2014).

1.5 Effects of dietary macronutrients across a developmental stage:

The earlier discussed DR evolutionary hypotheses and many of the experimental investigations of DR have focused on changes in adult life-history traits with dietary manipulation in adulthood. An individual's early-life condition, including resource availability, can have large consequences for both the individual's life-history traits in the current juvenile stage, as well as costs or benefits associated with later-life condition and life-history traits (reviewed in Lindström 1999; Metcalfe and Monaghan 2001, 2003). For example, the brood sizes of *Taeniopygia guttata* zebra finches were experimentally manipulated and small and large broods were given the same amount of egg-mixture food, resulting in fledglings of the smaller broods receiving higher amounts of food per individual (Kogel, 1997). Fledglings from large broods had lower survival both before and after independence, suggesting lowered feeding during development increased early-life, but also later-life mortality (Kogel, 1997). Such effects, including costs associated with catch-up growth with better conditions in adulthood, have been found across taxa (reviewed in Metcalfe and Monaghan 2001, 2003).

Due to having distinct developmental juvenile and adult stages, insects have been commonly used to study the effects of early-life diet on life-history trade-offs, both in terms of effects of early-life diet on current and adult traits

(reviewed in Boggs, 2009; Nestel et al., 2016). In holometabolous insects, larvae spend their time feeding to accumulate enough resources to metamorphose into adults successfully (reviewed in Mirth & Riddiford, 2007; Nestel et al., 2016). Larval resource acquisition has been proposed by an allocation framework to be allocated into somatic growth or maintenance, or stored to be used post-metamorphosis (reviewed in Boggs, 2009). These stored resources can then be used, along with resources from adult feeding, into reproduction and maintenance in adulthood (reviewed in Boggs, 2009).

Previous studies applying diet manipulation have found similar findings as proposed by this allocation framework (see examples in Boggs 2009; Nestel et al. 2016). In terms of macronutrient manipulation, measures of successful development into adulthood, including shortened development time and increased viability, are often highest on intermediate or high P:C (e.g. Rodrigues et al. 2015; Silva-Soares et al. 2017, but see e.g. Davies et al. 2018; Gray et al. 2018). Larval dietary P:C affects adult life-history traits as well, for example measures of adult reproduction increase with higher larval P:C (Rodrigues et al., 2015; Duxbury & Chapman, 2020). Larval P:C has more inconsistent effects on lifespan and ageing where measures of both peak at different P:C diets or there is no effect of larval P:C (e.g. Tu & Tatar, 2003; Runagall-McNaull et al., 2015; English & Uller, 2016; Davies et al., 2018).

What has been less studied is the effect of early-life diet on adult stress resistance, as studies often measure adult life-history traits post-juvenile feeding in benign conditions (but see De Block & Stoks, 2008; Andersen et al., 2010; Fellous & Lazzaro, 2010; Kelly & Tawes, 2013; Davies et al., 2018). For prevalent stressors in wild conditions, injury and infection, there is some evidence that larval diet may affect adult survival post-infection (Fellous & Lazzaro, 2010; Kelly & Tawes, 2013). As one study altered both nymphal and adult diet, and one measured aspects of adult immune responses without an infection, it has not been tested whether changing only larval diet affects adult survival post-infection. Therefore, it is currently unknown whether larval diet affects adult injury or infection survival

outcomes, and whether injury and infection alter the commonly seen adult life-history traits due to larval feeding. In this thesis, I will be applying injury and infection treatments to adult *D. melanogaster* raised on different P:C diets as larvae to test whether larval feeding affects adult post-infection life-history traits (see also sections 1.7 and 1.9).

1.6 Which diets do individuals choose?

According to optimal foraging theory, when given a choice between diets, individuals should forage and choose diets to maximise fitness (reviewed in Pyke et al., 1977; Stephens & Krebs, 1986; Simpson et al., 2004). Across taxa, this has largely been confirmed using various types of food manipulation or recording of food choice behaviour in the wild (reviewed in Stephens & Krebs, 1986; Pyke, 2019). For example, Redshank birds predated a predicted number of prey species depending on their size and availability of the two invertebrates in an estuary (Goss-Custard, 1977; see also discussion in Pyke et al., 1977). These earlier models focused more on overall energy intakes, however individuals have also been found to regulate food intake, and to choose an optimal level of over- or under-eating particular nutrients such as proteins and carbohydrates (reviewed in Simpson et al., 2004). In addition, when given a choice between two diets, individuals eat both diets selectively to reach an optimal intake, termed the intake target (reviewed in Simpson et al., 2004). These observations led to the application of optimal foraging models in terms of macronutrients (reviewed in Simpson et al., 2004; Raubenheimer et al., 2009).

Intake targets are intake ratios associated with maximum fitness, where diets away from this target are associated with lower fitness. In particular, GF studies (see section 1.2.1) have allowed direct comparisons between fitness landscapes and intake ratios from food choice tests. Intake ratios can be calculated from measuring individual's intakes when given a choice between pairs of complementary diets. From insect studies, adult individuals often choose P:C intakes which correspond to diets where reproduction measures peak (Lee et al. 2008; Fanson et al. 2009; Jensen et al. 2012; Malod et al. 2017, but see Harrison et

al. 2014). Intake targets can change across developmental stages, as for example in *D. melanogaster,* larvae choose intermediate P:C diets associated with faster development rate, suggesting they prioritise development time to avoid depletion of the diet source (Rodrigues et al., 2015), whereas adult *D. melanogaster* choose slightly higher P:C diets that maximise lifetime reproduction (Lee et al., 2008; Jensen et al., 2015). In *Nicrophorus vespilloides* burying beetles, although intake targets were similar, mature beetles prioritised energy intake, and younger beetles preferred protein, suggesting changes in feeding behaviour depending on the age of the individual (Al Shareefi & Cotter, 2019). In some cases, where males and females have different optimal P:C diets associated with reproduction, they choose the same diet, which is non-optimal for either (Maklakov et al. 2008; Jensen et al. 2015, but see Harrison et al. 2014; Malod et al. 2017).

Intake ratios are not fixed points and activities such as flying, or responding to infection, can affect them, suggesting variation in diet choice depending on multiple factors (reviewed in Simpson & Raubenheimer, 2012). Once infected, individuals often reduce total feeding, a common sickness behaviour, and choose diets associated with higher post-infection survival (reviewed in Hite et al. 2020, but see a few results from studies using immune challenges without a live infection, e.g. Aubert et al. 1995; Kelly and Mc Cabe Leroux 2020; Wilsterman et al. 2020). However, as depending on the host-pathogen pair, diets associated with higher post-infection survival vary (see section 1.7.1), food choice post-infection is required to be tested in a wide range of systems and with each pathogen to understand whether individuals choose diets associated with higher survival post-infection. In this thesis, I will test short-term food choice post-infection with *P. entomophila* to test whether food choice changes with this pathogen (see sections 1.9, 1.10 and 1.11).

1.7 Nutritional immunology and interactions of diet with injury:

1.7.1 Does macronutrient composition affect recovery from injury?

Poor diets in terms of decreased calories impair wound healing in rodents (Reiser et al., 1995; Hunt et al., 2012), and lizards (French et al., 2007). Similarly, protein malnutrition slows down wound healing in mice (Lim et al., 2006). Although injury subsets are often included in insect infection studies to determine whether the injury treatment given as part of systemic infection alters the measured traits, relatively few studies manipulating diets include an injury subset on different diets. The few studies that include an injury treatment often show diet has no effect on larval survival, either in terms of survival to pupation (Povey et al., 2009), or on survival when survival is measured for a few days post-infection, for larvae (Povey et al., 2014) or adult insects (Dinh et al., 2019; Sieksmeyer et al., 2021). In contrast, lower P:C increased *D. melanogaster* survival measured for 15 days post-injury, similar to infected individuals (Ponton et al., 2020). To date, no study has measured whether diet affects lifetime survival post-injury to assess whether injury affects DR responses (see section 1.3.3).

1.7.2 How does macronutrient composition affect life-history traits post-infection?

Nutrition influences disease outcomes across animal taxa, including humans (reviewed in Calder & Jackson, 2000; Coop & Kyriazakis, 2001; Katona & Katona-Apte, 2008; Ponton et al., 2011b, 2013; Becker et al., 2015). Nutritional immunology is a research field focusing on the interactive effects of diet and infection, and studying how different diets influence aspects of the host-pathogen relationship (reviewed in Ponton et al., 2011b, 2013). A common finding is that caloric restriction reduces survival post-infection, however, as caloric restriction reduces both overall and specific nutrients, it is unclear which component or components of diet are causing a reduction in lifespan post-infection (reviewed in Ponton et al., 2011b, 2013).

Studies manipulating macronutrient ratios or applying GF methods suggest that protein content is an important mediator of post-infection survival, where a

general finding is that individuals are often more likely to survive infection more on higher P:C diets (Peck et al., 1992; e.g. Lee et al., 2006; Povey et al., 2009, 2014; Cotter et al., 2019). There is evidence of the reverse directional effect of protein in diet, where survival post-infection is higher on lower P:C (e.g. Dinh et al., 2019; Ponton et al., 2020). This suggests pathogen-specificity in diet-mediated infection responses (see also Pike et al. 2019; Roberts and Longdon 2020, and diverging effects of diet supplementation on various host-pathogen interactions in the wild in Becker et al. 2015). Regardless of which P:C is associated with higher survival post-infection, often various measures of the immune response are higher on the diets associated with higher survival (e.g. Lee et al. 2006; Povey et al. 2009; Ponton et al. 2020).There is evidence that these immune responses peak at different P:C and calorie diets, and therefore that these responses trade-off between each other (Cotter et al., 2011, 2019). For example, in *Spodoptera littoralis* caterpillars, haemolymph phenoloxidase activity was higher at a higher carbohydrate content compared to where lysozyme activity was highest (Cotter et al., 2011). Individuals also often choose diets associated with higher post-infection survival (reviewed in Hite et al. 2020, but see Aubert et al. 1995; Wilsterman et al. 2020) (see section 1.6). In terms of reproduction post-infection, infection generally reduces reproduction (reviewed in Schwenke et al., 2016). Diet may affect these patterns as there is evidence that this typical reduction in reproduction is only apparent with higher P:C in *D. melanogaster* (Hudson et al., 2019).

Similar to measures of diet on injury recovery, many of the experiments measuring survival post-infection have been conducted in the larval stage (e.g. Lee et al., 2006; Povey et al., 2009; Cotter et al., 2019) or survival has been measured only for a few days post-infection (Kutzer & Armitage, 2016b; Lee et al., 2017; Dinh et al., 2019). For reproduction, only early-life reproduction has been measured (Hudson et al. 2019). Therefore, it is currently unknown how diet affects lifetime survival or reproduction post-infection, and so whether DR responses including the increase in lifespan change with infection (see section 1.2.1). In this thesis, I will

measure whether life-history trade-offs change with injury and infection, including measures of lifetime survival and reproduction (see section 1.9).

1.7.3 Why does diet alter infection outcomes?

It is not fully understood why diet affects post-infection survival. Protein has been suggested to be a limiting resource which is required in the immune response, or to repair damage caused by the pathogen (Lochmiller & Deerenberg, 2000; Lee et al., 2006). The host and pathogen share the same resources, and therefore altered nutrition can affect the growth of bacteria directly (Ponton et al., 2013; Cressler et al., 2014). For example, increased nutrition can increase pathogen growth by providing a greater availability of resources used by the pathogen to grow faster (Cressler et al., 2014). Increased protein can limit growth by increasing the growth medium solute concentration (osmolality), as found with *Xenorhabdus nematophila* bacterial infection in *S. littoralis* caterpillars (Wilson et al., 2020). As diet affects nutrient signalling pathways (see section 1.4), which are also affected by infection as studied in insects (reviewed in Ponton et al., 2013; Galenza & Foley, 2019), and pathogens have been found to affect aspects of these pathways (Chakrabarti et al., 2012), interactions between immune and diet responsive pathways may also explain survival differences with diet.

Diet has also been proposed to alter an individual's disease tolerance or resistance (reviewed in Ponton et al. 2013). Increased resistance is defined as when the pathogen growth is reduced, for example by faster clearing post-infection (reviewed in Kutzer & Armitage, 2016a; Lissner & Schneider, 2018; Martins et al., 2019). Increased tolerance is that the individual is able to tolerate the pathology of a given pathogen load, for example by repairing or preventing damage caused by the pathogen (reviewed in Kutzer & Armitage, 2016a; Lissner & Schneider, 2018; Martins et al., 2019). A common finding in insect studies measuring immune responses is that increased resistance is associated with the diet with higher post-infection survival (reviewed in Ponton et al. 2013), where many studies find lowered pathogen loads on diets associated with higher post-infection survival (e.g. Lee et al., 2017; Dinh et al., 2019; Wilson et al., 2020). This effect on increased resistance is

not universal, as several insect studies do not see changes in pathogen loads on different diets depending on the pathogen, and therefore suggest a role of increased tolerance due to an increase in survival post-infection outside of bacterial clearance (Ayres and Schneider 2009; Miller and Cotter 2018).

There are also several examples of diet effects on infection resistance in vertebrates. *Lithobates sphenocephalus* southern leopard frog tadpoles given a higher P:C diet had increased resistance in terms of lower presence of chytrid fungus post-infection exposure (Venesky et al. 2012), where resistance is considered as the ability to resist getting infected (reviewed in Kutzer & Armitage, 2016a). In sheep, protein supplementation increases resistance to various types of infections (reviewed in Coop & Kyriazakis, 2001). In mice, protein malnutrition increased the number of worms both in a primary and a secondary infection with *Heligmosomoides polygyrus* nematodes compared to mice given more protein (Ing et al., 2000). After a viral infection, protein malnourished mice had a lower clearance of virus and were more likely to die post-infection (Taylor et al., 2013). In the wild, food supplementation increased *Apodemus sylvaticus* resistance to *H. polygyrus,* which was confirmed in the laboratory (Sweeny et al., 2019). However, food supplementation had mixed effects on *A. sylvaticus* endoparasites depending on the time taken for a full lifecycle for the specific parasite (Díaz & Alonso, 2003), suggesting diet can have diverging effects on parasites in wild conditions.

Furthermore, both resistance and tolerance can be affected by diet. In *Serinus canaria* canary birds infected with *Plasmodium relictum* malaria, birds supplemented with protein and vitamins had increased clearance rate of the pathogen (increased resistance), however higher anaemia, suggesting a higher cost of infection (lower tolerance) (Cornet et al., 2014). One of two mouse strains infected with *Heligmosomoides polygyrus* nematodes on low protein diets had higher nematode loads (lower resistance) and increased intestinal permeability at this pathogen load (lowered tolerance) (Clough et al., 2016). For *Osteopilus septentrionalis* frogs infected with *Aplectana* sp. nematodes, frogs which

were given a higher number of crickets to eat, had higher resistance or tolerance depending on the stage of infection (Knutie et al., 2017b). Taken together, with evidence of host or pathogen specificity discussed earlier, this suggests that diet and infection have complex interactions on survival and other traits post-infection. Therefore, in this thesis, I will measure food choice and bacterial load and a measure of the immune response (AMP gene expression, see section 1.10) post-infection with *P. entomophila* infection to understand how diet affects these responses in this host-pathogen pair (see section 1.9 to 1.11).

1.8 Considering variation in DR responses, would DR still be applicable in human populations?

DR has been considered to be applied in humans to aid with an ageing society and to slow appearance of old-age pathologies (Speakman & Mitchell, 2011; e.g. Redman & Ravussin, 2011; Gray et al., 2018; Pignatti et al., 2020). Many studies in humans report some beneficial effects of DR on health (Walford et al., 2002; Redman & Ravussin, 2011; Fontana & Partridge, 2015; Pignatti et al., 2020). There is also extensive discussion about the considerable negative consequences of such applications, including physiological and psychological problems and concerns (reviewed in Dirks & Leeuwenburgh, 2006; Fontana et al., 2010; Redman & Ravussin, 2011; Fontana & Partridge, 2015). In addition, long-term CR is hypothesised to only increase lifespan by a few years (Speakman & Mitchell, 2011; reviewed in Redman & Ravussin, 2011). Intermittent fasting, where various formats of CR are followed for a certain number of hours or a number of days, may be a more applicable method of DR (reviewed in Mattson et al., 2017; de Cabo & Mattson, 2019; Duregon et al., 2021). However, there is some indication that people following intermittent fasting remain feeling hungry or otherwise are inconvenienced by the diet depending on the application (Mattson et al., 2017), requiring further study (see also discussion in Duregon et al., 2021).

Another more feasible method of increasing lifespan in human populations may be the reduction in protein or specific amino acid intakes, or decreasing P:C macronutrient intakes (reviewed in Pignatti et al., 2020). However, there is also

evidence that the particular source of protein may affect whether such changes will be beneficial in humans and that increased protein intake in old-age may be favourable (reviewed in Pignatti et al., 2020). Effects of macronutrient changes have not been extensively studied in humans and require more research prior to stating whether they are applicable to humans, and not associated with health problems (reviewed in Pignatti et al., 2020).

DR mimetics may be a more feasible translational approach of applying DR in humans (reviewed in Fontana et al., 2010; Speakman & Mitchell, 2011; Selman, 2014). DR mimetics are drugs or other interventions that mimic DR effects through manipulating metabolism or other associated pathways involved in DR responses, without directly changing diets (reviewed in Ingram et al., 2004; Dirks & Leeuwenburgh, 2006; Speakman & Mitchell, 2011). Many of these DR mimetics are focused on the nutrient-sensing pathways (see section 1.4) (reviewed in Ingram et al., 2004; Dirks & Leeuwenburgh, 2006; Speakman & Mitchell, 2011). Even if DR in the form of changing diets in human populations is unfeasible or associated with health problems, further understanding in which conditions DR extends lifespan will allow for inferences on the feasibility of DR mimetics (Selman, 2014). Although a very broad extension of the work involved in this thesis, to understand whether DR methods could potentially be used in a human context, in general a further understanding about whether DR extends lifespan with additional stressors of injury and infection, and whether early-life diet affects these later-life traits are needed (see sections 1.3, 1.5 and 1.9), and therefore the results from this thesis will add to our broad understanding of DR responses.

1.9 Thesis aims:

This thesis has the following aims:

1.9.1 Do additional stresses of injury and infection remove the lifespan benefit of DR, and are some diets better for *D. melanogaster* survival post-infection with *Pseudomonas entomophila* (see section 1.10)?

The NRH predicts that with additional stressors such as injury and infection, the lifespan extension from DR is removed, whereas the RRH makes no additional predictions based on the environment (see section 1.3). I will apply injury and infection stress treatments to adult female *Drosophila melanogaster* flies (see section 1.11) to test whether DR, in terms of changes in P:C ratios, extends lifespan only in the control unstressed flies. In addition, diet is known to alter infection outcomes (see section 1.7). I will measure how *P. entomophila* infection affects life-history trade-offs post infection, including which diets are associated with higher post-infection survival.

1.9.2 Does larval dietary macronutrient manipulation affect adult life-history traits and survival post-infection?

Early-life diet affects multiple adult life-history traits, however it is less well-known how the combination of early-life diet and infection affect adult life-history traits (see sections 1.5 and 1.7.1). I will apply larval dietary P:C manipulation and infect adult female flies to measure how larval diet affects adult post-infection life-history trade-offs.

1.9.3 Do infected *D. melanogaster* individuals have altered diet preference post-infection with *P. entomophila*?

Infected individuals reduce food intake and often prefer diets associated with higher post-infection survival (see sections 1.6 and 1.7.2). For *P. entomophila* infection in *D. melanogaster*, it is not known how infection affects food choice post-infection (see section 1.10). I will measure short-term food choice post-infection and injury treatments to measure whether food choice changes with *P. entomophila* infection.

1.9.4 Does diet affect host resistance or disease tolerance with *P. entomophila* infection?

Diet can affect host resistance (reduced pathogen loads on diet associated with higher survival) or disease tolerance (increased survival outside of changes in pathogen load) (see section 1.7). I will measure bacterial load and expression of

AMP genes associated with *P. entomophila* infection (see section 1.10.2) on two diets to determine whether bacterial loads and AMP gene expression change with diet.

1.10 *Drosophila melanogaster – Pseudomonas entomophila* host-pathogen system:

To achieve these aims, I will utilise the well-described *D. melanogaster – P. entomophila* host pathogen system (reviewed in Dieppois et al., 2015). Below, I outline why I use this system.

1.10.1 *D. melanogaster* immune system:

D. melanogaster immune and metabolism systems have many similarities to vertebrate systems (reviewed in Galenza & Foley, 2019), making it an ideal study system to explore the effects of altering diets on life-history trade-offs post-injury or infection. *D. melanogaster* immune response consists of innate responses, however they do not have an adaptive immune system (reviewed in Lemaitre & Hoffmann, 2007). Depending on the method of infection or the type of pathogen, *D. melanogaster* have various different host immune responses, including encapsulation, production of reactive oxygen species (ROS) via the phenoloxidase pathway, coagulation and melanisation (reviewed in Lemaitre & Hoffmann, 2007; Troha & Buchon, 2019).

An additional host response after pathogen recognition is the production of antimicrobial peptides (AMPs), which are produced in the fat body and then circulated in the haemolymph, or produced locally, for example in the gut with oral infection (reviewed in Hoffmann & Reichhart, 2002; Lemaitre & Hoffmann, 2007; Hanson & Lemaitre, 2020). The type of pathogen is recognised by the host and appropriate production of AMPs is upregulated through different signalling cascades. The type of peptidoglycan (PGN) found on the pathogen is recognised by the host, where Lys-type PGNs found mainly on gram-positive bacteria and fungi activate the Toll signalling cascade, whereas DAP-type PGNs found mainly on gram-negative bacteria and activate the Imd signalling cascade (reviewed in

Lemaitre & Hoffmann, 2007). Both signalling pathways can express some AMPs such as Drosomycin (De Gregorio et al., 2002). Although sets of AMPs are produced in response to infection, different AMPs or subsets of AMPs are specific to different types of bacterial infections and tightly regulated (Hanson et al., 2019b).

1.10.2 *Pseudomonas entomophila* bacterial entomopathogen:

P. entomophila is a gram-negative bacterial pathogen that was extracted from a wild *D. melanogaster* in Guadeloupe (Vodovar et al., 2005). *P. entomophila* is an entomopathogenic bacteria, as it is able to infect other insect orders, and it has been proposed to be an opportunistic pathogen inhabiting soil (reviewed in Dieppois et al., 2015). Individuals ingesting *P. entomophila* are suggested to die due to accumulation of bacteria in the gut, and due to damage caused by the bacteria and the host immune system in the gut, leading to gut damage and death (Chakrabarti et al., 2012; Dieppois et al., 2015). After ingesting *P. entomophila, D. melanogaster* larvae stop feeding due to a food uptake blockage (Vodovar et al., 2005; Liehl et al., 2006), but it is not currently known whether *P. entomophila* infection leads to a similar reduction in food uptake in adults. *Blatta orientalis* cockroaches systemically infected with *P. entomophila* did not stop feeding, but instead reduced feeding and chose a higher P:C intake compared to uninfected individuals (Sieksmeyer et al., 2021).

The *D. melanogaster* immune response to *P. entomophila* includes the production of ROS and production of AMPs (reviewed in Dieppois et al., 2015). Various Imd signalling cascade associated AMPs are produced both systemically, and with oral infection through feeding, locally in the gut (Vodovar et al., 2005; Liehl et al., 2006). As a pathogen-mediated defence strategy against these host responses, *P. entomophila* inhibits global translation by approximately 50% in the gut, so that AMP transcripts are not translated into functioning AMPs (Chakrabarti et al. 2012). However, this global inhibition also obstructs gut cell epithelium renewal, a process where the gut is repaired (Chakrabarti et al. 2012). Although *P. entomophila* inhibits translation of AMPs, this is not complete, and the induction

of Diptericin or Attacin A AMPs in otherwise Imd mutant flies increases host survival post-infection (Liehl et al. 2006). There is considerable genetic variation in susceptibility to *P. entomophila* infection, where from 140 lines of the *Drosophila melanogaster* genetic reference panel (DGRP) given the same oral infection dose, survival ranged from 0 to 100% dead when measured for three days post-infection (Bou Sleiman et al., 2015). While *P. entomophila* has been well-studied (reviewed in Dieppois et al., 2015), *P. entomophila* infection has not been studied in the context of understanding whether host diet alters life-history trade-offs or other host-pathogen traits post-infection in *D. melanogaster*.

1.11 Common experimental methods:

1.11.1 Fruit fly experimental outbred DGRP population:

In chapters 2 to 5, we used flies from an outbred population of *D. melanogaster*, created in the laboratory prior to being used here (see Appendix A). This population was created by crossing 113 DGRP lines (Mackay et al., 2012) in 100 pairwise crosses (consisting of two age-matched virgin females and two age-matched males from different DGRP lines; see Appendix A) in vials containing modified Lewis food (Lewis 1960, see Table 1.1, 1:6 P:C/14% protein diet). The first generation of the outcross was made by placing all offspring from these initial pairwise crosses in a population cage and allowing them to interbreed and lay eggs on fruit juice agar plates. These eggs were collected by pouring PBS solution on the plates and collecting the egg solution in a falcon tube, which was then deposited into bottles containing Lewis food, following the method of Clancy and Kennington (2001) for maintaining *Drosophila* populations at constant densities.

To generate the next generation, each month the emerged adult flies from these bottles were pooled into a population cage to lay eggs following the same method of Clancy and Kennington (2001) (more information in Appendix A). In this way, the outcrossed DGRP population was housed in plastic bottles and outbred for 19 non-overlapping generations of complete outcrossing in 12 h light:dark cycles, at 25 °C (±1 °C) and constant humidity prior to using in the work in this thesis. Each

chapter will outline which specific generation of the outcross was used. Many of the original DGRP lines carry the bacterial endosymbiont Wolbachia (Mackay et al. 2012). The DGRP panel in the laboratory was cleared of Wolbachia over seven years prior to the creation of the outcrossed population.

1.11.2 Experimental diets:

In each chapter, flies were maintained on diets varying in protein to carbohydrate (P:C) ratios. There were 10 diets in total, and each chapter indicates if all or a subset of diets were used. These diets were made by altering the mass of yeast or sugar added to a modified Lewis food recipe (Lewis, 1960, Table 1.1). One of the main differences to the original Lewis food recipe is the replacement of dextrose and sucrose with brown sugar in our diets (Lewis, 1960). The 10 diets were a span of P:C values (from 1:26 to 2.5:1 P:C), where protein restriction has previously been shown to extend lifespan (Lee, 2015). Yeast and sugar are approximately isocaloric, so P:C ratios can be altered without altering the energy content of the diet by replacing yeast with sugar (Mair et al., 2005). Prior to any tests as detailed in the chapter methods, flies were housed on modified Lewis food with 1:6 P:C (14% protein diet).

Protein percentages and P:C values incorporate protein and carbohydrate values from maize (Table 1.1). Protein percentages are shown as the percentage of total protein from the total amount of yeast, sugar and maize in the diet, whereas the P:C ratio is the total protein compared to the total carbohydrate in the diet (Table 1.1). Yeast contains various micronutrients and carbohydrates outside of protein (Simpson & Raubenheimer, 2009; Lee, 2015), however here yeast is considered only as a protein source due to lack of direct quantification of dietary protein and carbohydrate in the yeast used (Table 1.1). Similarly, brown sugar is only considered as a carbohydrate source (Table 1.1). Protein and carbohydrate for maize is estimated from Doves Farm (Doves Farm, 2021, 70% carbohydrate, 9.1% protein, note however that the nutritional information has since changed to 77.4% carbohydrate and 5.1% protein, so the final values might change slightly, e.g. diet with no sugar added would be 63.9% protein or 2.2:1 P:C).

To facilitate easier comparison to previous studies where protein contributed by yeast is known, an alternate potential P:C composition of the diets is provided in Table 1.2. Here, protein and carbohydrate from yeast is estimated for Fermipan Red Dried Yeast from Turner Price (Turner Price, 2021, 50% protein, 40% carbohydrate) and for brown sugar from Food Data Central (U.S. Department of Agriculture Agricultural Research Service, 2020, 0.1% protein, 98.1% carbohydrate). It should be noted that many previous studies have manipulated diets in very different ways, which may make comparisons outside of P:C changes between studies even more difficult (see also discussion in Lesperance & Broderick, 2020). Such differences include the application of chemically defined diets with protein not from yeast but from sodium caseinate (Lee, 2015; Kim et al., 2019, 2020) or amino acids (Jensen et al., 2015), some studies apply liquid instead of solid diets (e.g. Lee et al., 2008; Jensen et al., 2015; Than et al., 2020), and many studies do not include cornmeal in the diet (e.g. Skorupa et al., 2008; Rodrigues et al., 2015). For this thesis, when discussing P:C or protein percentages, values from Table 1.1 will be used.

Table 1.1: Ten diets and their corresponding P:C ratios with additional information of each added ingredient. The standard modified Lewis food (Lewis 1960) and associated P:C ratio is in bold. The P:C ratios (rounded to one decimal place) incorporate the protein and carbohydrate contributed by maize (estimated from Doves Farm (Doves Farm, 2021, 70% carbohydrate, 9.1% protein, note however that the nutritional information has since changed to 77.4% carbohydrate and 5.1% protein, so the final values might change slightly, e.g. diet with no sugar added would be 63.9% protein or 2.2:1 P:C). Protein percentages show the total protein of the diet out of the added yeast, brown sugar and cornmeal (rounded to one decimal place). Although yeast is also composed of carbohydrates, lipids and micronutrients (Simpson & Raubenheimer, 2009; Lee, 2015), here yeast is only considered as a source of protein, as we did not quantify the actual protein and carbohydrate present in the yeast used in our laboratory. Similarly, brown sugar is only considered as a carbohydrate source. Two baseline ratios were made with no addition of yeast (1:26 P:C, 3% protein) or sugar (2.5:1 P:C, 65% protein) (see table on next page).

P:C ratio	Protein (%)	Yeast (g)	Sugar (g)	Maize (g)			Agar (g)	Nipagin (ml)	dH_2O (l)
				Total	Of which carbohydrate	Of which protein			
1:25.6	3	0.0	675.0	415	290.5	37.8	41.2	90	6
1:16.0	5	21.3	653.7	415	290.5	37.8	41.2	90	6
1:8.0	10	73.7	601.3	415	290.5	37.8	41.2	90	6
1:5.7	**14**	**112.5**	**562.5**	**415**	**290.5**	**37.8**	**41.2**	**90**	**6**
1:4.0	18	162.9	512.1	415	290.5	37.8	41.2	90	6
1:2.0	31	296.7	378.3	415	290.5	37.8	41.2	90	6
1:1.0	46	463.9	211.1	415	290.5	37.8	41.2	90	6
1.5:1	55	564.2	110.8	415	290.5	37.8	41.2	90	6
2.0:1	61	631.1	43.9	415	290.5	37.8	41.2	90	6
2.5:1	65	675.0	0.0	415	290.5	37.8	41.2	90	6

Table 1.2: Alternate estimates of the ten diets and their corresponding P:C ratios with additional information of each added ingredient. Here, protein (P) and carbohydrate (C) from yeast is estimated for Fermipan Red Dried Yeast from Turner Price (Turner Price, 2021, 50% protein, 40% carbohydrate), for brown sugar from Food Data Central (U.S. Department of Agriculture Agricultural Research Service, 2020, 0.1% protein, 98.1% carbohydrate) and for maize from Doves Farm (Doves Farm, 2021, 70% carbohydrate, 9.1% protein, note however that the nutritional information on the website has since changed to 77.4% carbohydrate and 5.1% protein, so the final values might change slightly, e.g. diet with no sugar added would be 32.9% protein or 1:1.7 P:C). The standard modified Lewis food (Lewis 1960) and associated P:C ratio is in bold. The P:C ratios (rounded to one decimal place) incorporate the protein and carbohydrate contributed by maize, yeast and brown sugar. Two baseline ratios were made with no addition of yeast (1:24.8 P:C, 3.5% protein) or sugar (1:1.5 P:C, 34.4% protein) (see table on next page).

P:C ratio	Protein (%)	Yeast (g)			Sugar (g)			Maize (g)			Agar (g)	Nipagin (ml)	dH_2O (l)
		Total	P	C	Total	P	C	Total	P	C			
1:24.8	3.5	0.0	0.0	0.0	675.0	0.7	662.2	415	37.8	290.5	41.2	90	6
1:19.2	4.5	21.3	10.7	8.5	653.7	0.7	641.3	415	37.8	290.5	41.2	90	6
1:12.1	6.9	73.7	36.9	29.5	601.3	0.6	589.9	415	37.8	290.5	41.2	90	6
1:9.4	**8.7**	**112.5**	**56.3**	**45.0**	**562.5**	**0.6**	**551.8**	**415**	**37.8**	**290.5**	**41.2**	**90**	**6**
1:7.2	11.0	162.9	81.5	65.2	512.1	0.5	502.4	415	37.8	290.5	41.2	90	6
1:4.2	17.1	296.7	148.4	118.7	378.3	0.4	371.1	415	37.8	290.5	41.2	90	6
1:2.5	24.8	463.9	232.0	185.6	211.1	0.2	207.1	415	37.8	290.5	41.2	90	6
1:2.0	29.4	564.2	282.1	225.7	110.8	0.1	108.7	415	37.8	290.5	41.2	90	6
1:1.7	32.4	631.1	315.6	252.4	43.9	0.0	43.1	415	37.8	290.5	41.2	90	6
1:1.5	34.4	675.0	337.5	270.0	0.0	0.0	0.0	415	37.8	290.5	41.2	90	6

1.11.3 Injury and Infection stress treatments:

In each chapter, flies were exposed to one of three stress treatments: control, injury or infection (unless otherwise stated). The control treatment involved handling flies under CO_2 anaesthetisation and then transferring these to a new vial containing the relevant diet, as CO_2 treatment can affect behavioural and egg laying in *D. melanogaster* (Bartholomew et al., 2015; MacMillan et al., 2017). The injury treatment involved the same protocol, however an enamelled pin was dipped in sterile LB broth and used to pierce the pleural suture under the left wing. For the infection treatment, the pin was dipped in a *Pseudomonas entomophila* bacterial broth from an overnight culture in LB at 30 °C (Dieppois et al., 2015; Troha & Buchon, 2019). Additional details of the infection procedure completed in each chapter are outlined in each chapter methods section separately.

1.11.4 Bacterial load (colony forming units, CFU/ml) measurement:

In chapters 3 to 5, bacterial load of flies (alive at the point of sampling) was analysed by counting colony forming units (CFU/ml) following Gupta et. al (2017). Flies were individually placed in 1.5 ml Eppendorf tubes (but see Chapter 3). Flies were first surface sterilised by adding 100 µl of 75% ethanol for 30-60 seconds, then the ethanol was discarded and the flies were washed twice in 100 µl of distilled water. 5 µl of this second wash was plated on LB plates to confirm that surface sterilisation was successful (no bacteria grew on plates incubated in 30 °C for 24 to 48 hours). Any flies which failed this step were not considered in the analysis. The flies were centrifuged at 12 G for 1 minute after which the distilled water was removed. 200 µl of LB broth was added to each Eppendorf, and for about a minute flies were homogenised using a pestle motor mixer. 100 µl of this liquid was placed into a 96 well plate and five 1:10 serial dilutions in LB broth were completed. 5 µl of these dilutions were plated on Pseudomonas isolating agar, incubated at 30 °C, and colonies were counted 24 hours later and checked again at 48 hours. The lowest dilution with colonies present was used to calculate the number of colonies present in the initial volume of homogenate.

Chapter 2:

Testing evolutionary explanations for the lifespan benefit of dietary restriction in fruit flies (*Drosophila melanogaster*)

As published in (journal article; previous associated pre-print; and data and associated script):

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Savola, E., Montgomery, C., Waldron, F. M., Monteith, K. M., Vale, P., & Walling, C. (2021). Testing evolutionary explanations for the lifespan benefit of dietary restriction in fruit flies (*Drosophila melanogaster*). *Evolution*, 75(2), 450-463. https://doi.org/10.1111/evo.14146

Savola, E., Montgomery, C., Waldron, F. M., Monteith, K., Vale, P., & Walling, C. (2020c). Testing evolutionary explanations for the lifespan benefit of dietary restriction in *Drosophila melanogaster*. *BioRxiv*, 1–39. https://doi.org/10.1101/2020.06.18.159731

2.1 Abstract:

Dietary restriction (DR), limiting calories or specific nutrients without malnutrition, extends lifespan across diverse taxa. Traditionally, this lifespan extension has been explained as a result of diet-mediated changes in the trade-off between lifespan and reproduction, with survival favoured when resources are scarce. However, a recently proposed alternative suggests that the selective benefit of the response to DR is the maintenance of reproduction. This hypothesis predicts that lifespan extension is a side effect of benign laboratory conditions, and DR individuals would be frailer and unable to deal with additional stressors, and thus lifespan extension should disappear under more stressful conditions. We tested this by rearing outbred female fruit flies (*Drosophila melanogaster*) on 10 different protein:carbohydrate diets. Flies were either infected with a bacterial pathogen (*Pseudomonas entomophila*), injured with a sterile pinprick or unstressed. We monitored lifespan, fecundity and measures of ageing. DR extended lifespan and reduced reproduction irrespective of injury and infection. Infected flies on lower protein diets had particularly poor survival. Exposure to infection and injury did not substantially alter the relationship between diet and ageing patterns. These results do not provide support for lifespan extension under DR being a side effect of benign laboratory conditions.

2.2 Introduction:

Nutrition has long been of interest in the field of ageing research, particularly due to its potential applications to an ageing human population (reviewed in Bertozzi et al., 2016; Redman & Ravussin, 2011; Speakman & Mitchell, 2011). Dietary restriction (DR), the limitation of a particular nutrient or the overall caloric intake without malnutrition, has been shown to extend lifespan and delay ageing across a range of organisms (reviewed in Mair & Dillin, 2008). Its prevalence and taxonomic diversity suggests the response is evolutionarily conserved and acts via conserved mechanisms (reviewed by Fontana et al., 2010). As such, a large body of research has focused on using the DR paradigm to try to understand the mechanisms underlying variation in ageing and lifespan (e.g. Gems and Partridge 2012; Fontana and Partridge 2015; Gibbs and Smith 2016). However, the evolutionary basis of the response has been much less well investigated (Zajitschek et al., 2016; Raubenheimer et al., 2016; Travers et al., 2020; Regan et al., 2020; Moatt et al., 2020). This is surprising given that knowledge of the evolutionary basis of the DR response is important to understanding under what conditions it may be applicable in human health. Here we test the two main evolutionary explanations for lifespan extension under DR, which make contrasting predictions about how this response should vary across environments.

The predominant evolutionary explanation, termed the resource reallocation hypothesis (RRH) (Adler & Bonduriansky, 2014; Regan et al., 2020), explains the observed DR response as an adaptive shift in relative investment of resources into survival versus reproduction (Kirkwood, 1977; Shanley & Kirkwood, 2000; Adler & Bonduriansky, 2014). A food shortage signals a sub-optimal environment, where the number and survival probability of any offspring produced is likely to be low (Holliday, 1989; Shanley & Kirkwood, 2000). Under such conditions, an individual could maximise fitness by temporarily delaying reproduction and instead investing resources into survival and somatic maintenance. Once food availability returns, the individual could then maximise fitness by investing resources back into reproduction. By maintaining individuals

chronically on low food, ageing rates decrease and the individual lives longer (Holliday, 1989; Shanley & Kirkwood, 2000). The RRH requires a trade-off between investing resources into reproduction versus somatic maintenance (Holliday, 1989) and that the response evolved in an environment which fluctuates between low and high food availability (Adler & Bonduriansky, 2014).

In contrast to the predictions of the RRH, some studies suggest that survival and reproduction can be uncoupled under DR (Flatt, 2011). In addition, wild systems have much higher levels of extrinsic mortality than laboratory conditions (for example, from predators or disease), potentially making an individual less likely to live long enough to benefit from delayed reproduction (Adler & Bonduriansky, 2014). These observations have been used to suggest that improved survival may not be the selective benefit of the DR response (Adler & Bonduriansky, 2014). Instead, another hypothesis proposes that the selective benefit of the DR response is through its effect on immediate reproduction (Adler & Bonduriansky, 2014), termed the nutrient recycling hypothesis (NRH) (Regan et al., 2020). This hypothesis is based on the general finding that DR results in the inhibition of nutrient sensing pathways, e.g. TOR and IIS pathways (Adler & Bonduriansky, 2014). Inhibition of these pathways disinhibits (upregulates) nutrient recycling mechanisms such as apoptosis (James et al., 1998) and autophagy (Hansen et al., 2008; Kenyon, 2010; Fontana et al., 2010, both reviewed in Longo & Fontana, 2010). The NRH suggests that apoptosis and autophagy allow the organism to use stored nutrients from cells whilst limiting the number of cells (Adler & Bonduriansky, 2014). The individual can use available resources more efficiently, with a possible lower resource requirement for reproduction (Adler & Bonduriansky, 2014).

The NRH posits that lifespan extension under DR is an artefact of laboratory conditions. Upregulation of apoptosis and autophagy may promote survival and limit rates of ageing due to protecting against common laboratory causes of death, such as cancer or other old age pathologies (Zhang & Herman, 2002; Spindler, 2005; Salomon & Jackson, 2008; Longo & Fontana, 2010; Adler & Bonduriansky,

2014). However, the limit on cell numbers and cellular growth rate may also limit the ability of individuals under DR to respond to additional stresses (Adler & Bonduriansky, 2014), with the prediction that DR would not extend lifespan in the wild (Adler & Bonduriansky, 2014). Thus, in contrast to the RRH, there is a clear prediction from the NRH that the addition of stressors, particularly injury and infection, should result in the removal or even reversal of the lifespan benefit of DR (Adler & Bonduriansky, 2014).

The effect of DR has been subject to relatively few studies in the context of injury and infection stress. In terms of injury stress, decreased calorie intake slows down wound repair in both rodents and reptiles (Reiser et al., 1995; Reed et al., 1996; French et al., 2007; Hunt et al., 2012). However, studies manipulating both overall calories and macronutrient content suggest that the main driver of the DR response, particularly in insects, is macronutrient ratio, with low protein and high carbohydrate diets leading to longer lifespans (e.g. Le Couteur et al., 2016; Kwang Pum Lee et al., 2008; Nakagawa et al., 2012; Simpson & Raubenheimer, 2009). In terms of infection stress, evidence for protein to carbohydrate (P:C) ratio effects on proxies of survival after infection are mixed. In infected caterpillars, higher protein increases performance, measured as the product of weight gain and survival to pupation (Lee et al., 2006; Povey et al., 2009, 2014), and lengthens the time to death for caterpillars dying post-infection prior to pupation (Cotter et al., 2019; Wilson et al., 2020). In adult fruit flies (*Drosophila melanogaster*), higher protein increased survival 24 hours post-infection with bacterial infection (Kutzer et al., 2018) and higher protein as extra yeast on top of food increased the number of days alive post-infection with a fungal pathogen (Le Rohellec & Le Bourg, 2009). In contrast, higher protein decreased survival measured up to 160 hours post-infection (Lee et al., 2017), 16 days post-infection in *D. melanogaster* (Ponton et al., 2020), and decreased survival 9 days post-infection in Queensland fruit flies (*Bactrocera tryoni*) (Dinh et al., 2019). However, to date none of these experiments have directly measured the key trait of lifetime survival. Additionally, studies often only use a small number of diets (Le Rohellec & Le Bourg, 2009; Lee et al., 2017;

Kutzer et al., 2018; Dinh et al., 2019; Ponton et al., 2020), or manipulate both P:C and calories at the same time (Le Rohellec & Le Bourg, 2009; Lee et al., 2017; Kutzer et al., 2018), making it hard to disentangle which aspect of the diet is affecting survival with injury or infection. Furthermore, no experiments have directly compared the effect of multiple diets on lifetime survival and reproduction in control, injured and infected individuals and thus tested the alternative predictions of the current evolutionary explanations of the DR response.

Here we address this gap in our knowledge by testing the contrasting predictions of the current evolutionary explanations of the DR response by including additional stressors of injury and infection to dietary restricted *D. melanogaster.* We achieved DR by altering the P:C ratio of food (e.g. Lee et al., 2008; Jensen et al., 2015) and thus throughout use the term protein restriction, although we acknowledge this also means the associated increase in carbohydrate, and changes in lipids and micronutrients. We measured lifespan, reproduction, and ageing measures, specifically the maintenance of gut integrity and climbing ability. These measures of ageing are often used to track treatment specific declines in function (e.g. Grotewiel et al., 2005; Martins et al., 2018) and allow us to measure whether ageing is delayed with DR under all stress treatments. We predict that if the RRH explains DR responses, all treatments would see the usual pattern of DR, where decreasing protein increases survival up to a point and then survival declines again due to malnutrition (see review Mair & Dillin, 2008). Regardless of the stress treatment, reproduction would increase with increasing protein and ageing would be delayed with lower protein. If the NRH explains DR responses, we would expect to see that with injury and infection, the lifespan increase expected under DR would disappear and injured and infected flies would not have the usual hump shape response of lifespan to decreasing protein in the diet. In addition, infected or injured individuals would not show delayed ageing with DR. Only the control group with no stress treatment would show the usual DR responses.

2.3 Methods:

2.3.1 Fly stocks and maintenance conditions:

From the 20th overlapping generation of the outbred population of *D. melanogaster* (as outlined in Chapter 1, section 1.11.1, and Appendix A), 4 µl of egg solution were placed into 20 plastic vials with modified Lewis food (see Table 1.1, Chapter 1, section 1.11.2, 14% protein, 1:6 P:C). After one generation, the adults were split into 50 vials, and to 60 vials from the second generation onwards. To create each generation, adults were transferred to new vials and allowed to lay eggs for two days before removal. Flies used for the experiment were offspring of the fifth generation from this protocol. The DGRP outcrossed population tested negative for common *Drosophila* laboratory viruses using primers described in Webster et al. (2015) with RT-PCR (unpublished data).

2.3.2 Experimental methods:

Adults of the fifth generation were density controlled (10 females/vial) to minimise subsequent variation in larval densities across vials, which can affect adult life-history traits (Graves & Mueller, 1993). Mated females were allowed to lay eggs for two days before removal. Vials were checked daily for adult eclosion. Flies were then maintained in vials for five days after adult eclosion began to allow mating to occur after which mated female flies from over 30 of these vials were transferred into the experiment following handling under CO_2 anaesthetisation. At this point, individual flies were singly housed on one of the ten diet treatments for the first experimental day (see below). On experimental day 2, flies from each diet treatment were assigned to one of three stress treatments: control, injury or infection (see below). There were 20 replicate flies per diet and stress treatment combination (20 individuals x 3 treatments x 10 diets = 600 flies in total). Females from one of the 30 vials were included across diet and stress treatments to account for some of the potential variation from larval or adult environment.

2.3.3 Diet treatments:

For the adult lifespan of each fly, flies were maintained on one of ten diets varying in protein to carbohydrate (P:C) ratio (Chapter 1, section 1.11.2 and Table 1.1 & 1.2), where the diets were dyed using a food dye (brilliant blue FCF E133, 3 g per 6 litres).

2.3.4 Stress treatments:

Stress treatments were applied as described in Chapter 1, section 1.11.3. To avoid lethal or negligible doses, an OD of 0.005 of *P. entomophila* culture was used, as determined in a previous pilot study (unpublished data).

2.3.5 Survival and fecundity measures:

Individuals were followed for life with survival scored daily. For the first two weeks of the experiment, individuals were tipped into fresh vials daily and afterwards every second day, with eggs (hatched and unhatched) counted when tipped. Any additional eggs in the vial were counted if a fly died on a day without a scheduled egg count. Diets and stress treatments were randomised across trays and trays were moved around the incubator daily to minimise microclimate effects.

2.3.6 Measures of physiological ageing:

2.3.6.1 Gut deterioration (smurf) assay:

In *D. melanogaster,* and other species (Martins et al., 2018), physiological ageing is associated with increased gut permeability, which can be assessed by feeding flies food with a blue dye and observing a change in body colour if the dye leaks from the gut (Rera et al., 2011). All diets included a blue food dye following Rera et al. (2011) at a lower concentration (3 g per 6 litres) to allow individuals to be scored for the "smurf" phenotype with age (Rera et al., 2011). Flies were scored as smurfs if the full body was blue, rather than just a small amount in the abdomen (Rera et al., 2011).

2.3.6.1 Negative geotaxis (NG) assay:

As flies age, their escape response declines and this deterioration can be measured with a negative geotaxis (NG) assay (e.g. Arking & Wells, 1990; Gargano et al., 2005; Linderman et al., 2012). NG was measured once every two weeks from week three, with a method modified from Arking and Wells (1990, see Appendix B). Briefly, flies were individually tipped into clean vials, knocked down to the bottom and then scored for whether they climbed to 4 cm on the vial within 60 seconds (1 for passing line, 0 for not passing the line).

2.3.7 Statistical methods:

The data were analysed using R software, version 3.5.2 (R Core Team, 2014) and graphs were drawn using ggplot2 (Wickham, 2016). Diet was analysed as a continuous covariate, representing the percentage of protein (Table 1.1, Chapter 1, 1.11.2), and its quadratic effect to allow for non-linear effects, while stress treatment was analysed as a categorical fixed effect. These and their interactions were included in all models. When reporting the results of the full models, the reported main effect represents the posterior mean and associated credible intervals for the baseline of control unstressed flies. For interactions, posterior means and associated credible intervals are the differences in slope for the specific effect in comparison to the control unstressed baseline (main effects in the model). To avoid scaling errors, all variables were standardised to a mean of zero with a standard deviation of one. This was done separately for each test due to different sample sizes. We used the R package MCMCglmm (Hadfield, 2010) for all models using a Poisson error distribution, unless otherwise stated. Further details are included in supporting information.

For all models, full models without model simplification are used due to the hypotheses specifically asking about the significance of the terms, e.g. whether infection and injury have significantly different estimates suggesting these stress treatments alter the significant patterns in the control flies (see also e.g. Whittingham et al., 2006; Forstmeier & Schielzeth, 2011). In addition, using full

models without model simplification, especially in an experimental set up where sample sizes are not as limiting as compared to observational studies, allows the avoidance of problems associated with model simplification such as multiple hypothesis testing, the assumption that a single model is the most appropriate choice and the possibility that removal of small but non-significant effects can bias the remaining estimates (see e.g. Whittingham et al., 2006; Forstmeier & Schielzeth, 2011; Hegyi & Laczi, 2015).

We used the R Survminer package (Kassambara & Kosinski, 2018) to graph Kaplan-Mayer curves for each stress treatment with diet as a factor. Our survival data violated the Cox proportional hazards assumptions, so we used an event history model where survival was analysed as a binomial trait, with each day scoring a fly as 0 for alive or 1 for dead, following Moatt et al. (2019). We included random effects of individual identity to account for repeated measures and experimental day to account for variation in survival across days. To confirm these results, we also analysed lifespan (see Appendix B for details).

Due to higher mortality closer to post-infection treatment with lower mortality after this time point, the survival data was analysed with the full dataset separated into individuals that survived either pre- and post-10 days post-infection (see Appendix B for details and Figure S2.5). Similar models to the full dataset were completed, where models for individuals that did not survive up to 10 days post-infection treatments only included infected individuals due to lack of dead flies in control and injury groups (see Appendix B, Table S2.4). The event history binomial models and Cox proportional hazards (with proportional hazards assumptions not met) included all individuals that survived post-10 days as censored data points for day 10. An event history binomial model including the full dataset with flies categorised as dying before day 10 or after day 10 was used to analyse the changes in diet patterns, with this categorisation and its interactions with mean centered protein and its squared term were included.

We analysed lifetime reproduction and additionally, to remove the effect of lifespan, we included mean centered lifespan in a separate model. For easier

comparisons to other studies, early egg production was analysed (days 2-7, as first day counts were similar across diets (Appendix B, Figure S1.1)). To investigate reproductive senescence, daily egg counts were analysed with age (in days) and its squared term as fixed effects and with mean centred lifespan as a fixed effect, to control for selective disappearance (Van de Pol & Verhulst, 2006), and a random effect of individual identity was included. The binomial variable for appearance of a blue body (1 for smurf, 0 for none) was analysed with a categorical model. Negative geotaxis was analysed as a binomial variable (1 for passing test, 0 for not) with a categorical model. Data and associated script are available on the Dryad repository (Savola et al., 2020a).

2.4 Results:

2.4.2 Survival and lifespan:

Analysing the survival data with an event history binomial model, the improvement in survival with reduced protein from very high protein levels (i.e. the classical DR response in *D. melanogaster*) did not differ across treatments, and survival was maximised at relatively similar intermediate protein levels across treatments (Figure 2.1 & 2.2; Table 2.1; Protein^2 = 0.48 (95% credible interval (CI) = 0.26 to 0.71), p = <0.001; Injury:Protein^2 = -0.16 (95% CI = -0.51 to 0.18), p = 0.36; Infection:Protein^2 = -0.01 (95% CI = -0.33 to 0.30), p = 0.99). There was a significant interaction between protein and stress treatment, with survival increasing more rapidly from low to intermediate protein levels for the infected treatment than for any other treatment (Figure 2.1 & 2.2; Table 2.1; Protein = 0.02 (95% CI = -0.13 to 1.17), p = 0.82; Infection:Protein = -0.31 (95% CI = -0.57 to -0.10), p = 0.004). This difference may be due to the low survival of infected individuals on low protein diets (Figure 2.1).

Stress treatment had a significant effect on survival, with individuals exposed to infection having a greater risk of death compared to control individuals for the duration of the experiment (Figure 2.2; Table 2.1; Infection = 0.66 (95% CI = 0.28 to 1.10) p = 0.002). There was no significant difference between injury and control treatments (Figure 2.2; Table 2.1; Injury = 0.14 (95% CI = -0.32 to 0.57), p = 0.54). Analysing lifespan (in days) showed very similar patterns to the binomial survival analysis (Appendix B, Figure S2.2 and S2.3; Table S2.1). Although our survival data violated the Cox proportional hazards model assumptions (see Appendix B), the results from a Cox proportional hazards model were similar to those from the event history and lifespan models (Appendix B, Figure S2.4; Table S2.2).

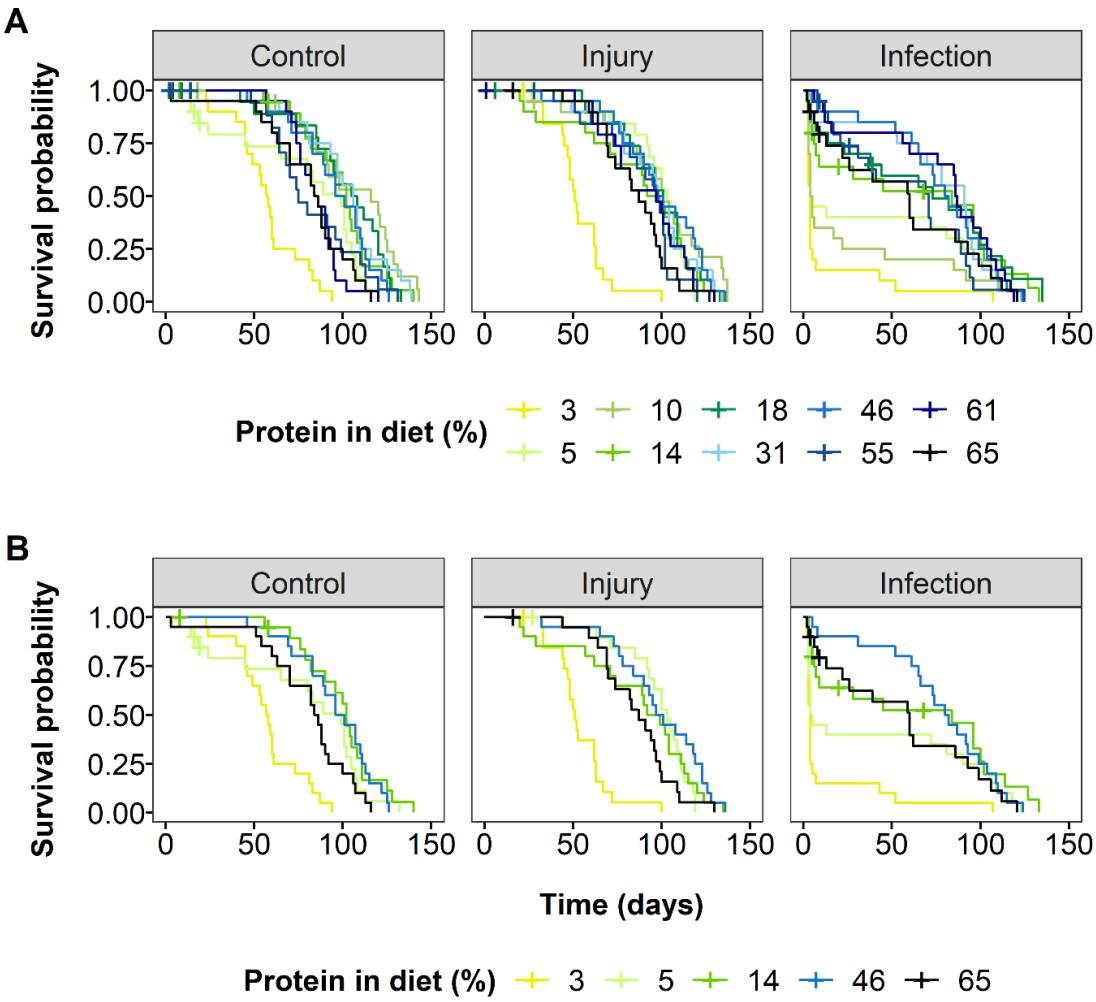

Figure 2.1: Effects of protein restriction on survival of flies infected with a bacterial pathogen ("Infection"), injured by a pinprick ("Injury") or with no treatment ("Control"). Survival is shown as Kaplan-Meier curves for each stress treatment and protein restriction diets (A). For ease of interpretation, a subset of protein restriction diets is shown in (B) to illustrate the effects of protein restriction with low (yellow and green lines), intermediate (light blue lines) and high protein content (dark blue and black lines). Survival was maximized on intermediate protein across all stress treatments, as survival was poor on low (yellow line) and high protein diets (black line). Plus signs (+) indicate censored data points.

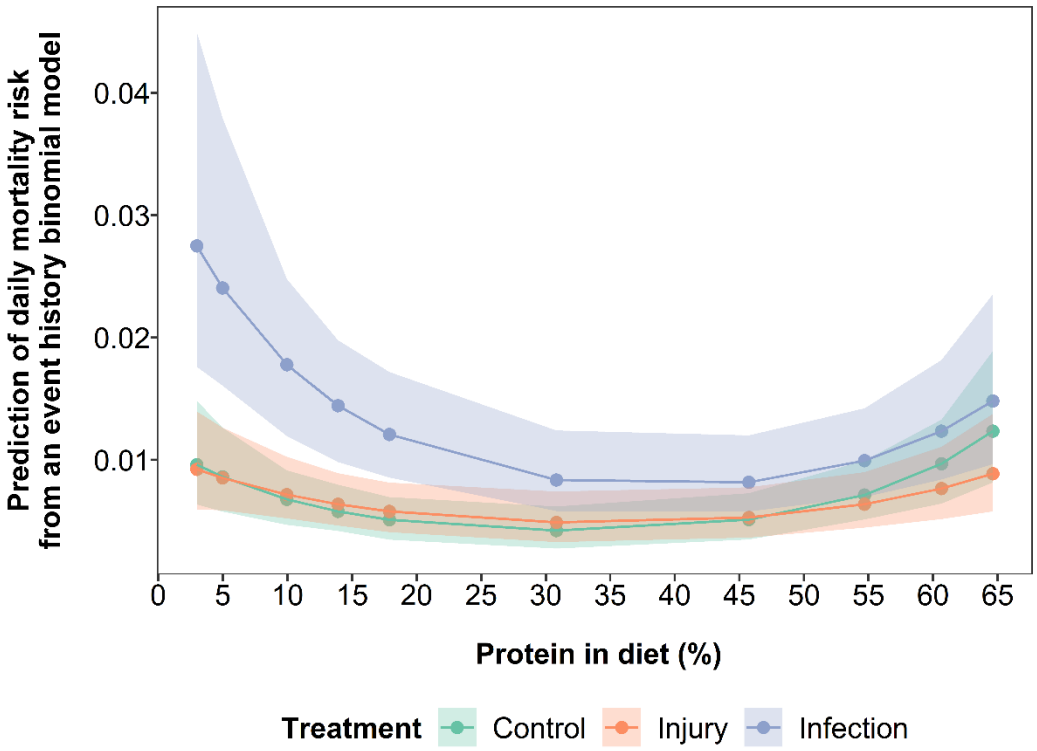

Figure 2.2: Model predictions from an event history binomial model for the effect of protein restriction on mortality risk per day of flies infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines). In the binomial model, for each day each fly was coded as 0 for alive and 1 for dead. Protein and protein^2 are mean centered to standard deviation of 1. Shaded areas are 95% credible intervals. Please note that the predictions are not curved as parabolas, as the model predictions have been transformed back to the data scale.

Table 2.1: Model summary of effects of protein restriction and stress treatments on mortality risk per day from an event history binomial model. In the binomial model, per each fly for each day, 0 coded for flies alive and 1 for dead. Protein and protein^2 are mean centered to standard deviation of 1. The model included random effects of Individual ID (posterior mean = 0.03 (95% credible interval (CI) = 7.56×10^{-10} to 0.11), effective sample size = 1013) and Experimental day (posterior mean = 2.38 (95% CI = 1.61 to 3.25), effective sample size = 1000). Significant results below significance level $\alpha = 0.05$ are bolded.

	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
Intercept	**-5.46**	**-5.89**	**-5.08**	**1000**	**<0.001**
Injury treatment	0.14	-0.32	0.57	1000	0.54
Infection treatment	**0.66**	**0.28**	**1.10**	**1000**	**0.002**
Protein	0.02	-0.13	1.17	1000	0.82
Protein^2	**0.48**	**0.26**	**0.71**	**1111**	**<0.001**
Injury:Protein	-0.08	-0.30	0.15	1000	0.45
Infection:Protein	**-0.31**	**-0.57**	**-0.10**	**1000**	**0.004**
Injury:Protein^2	-0.16	-0.51	0.18	1000	0.36
Infection:Protein^2	-0.01	-0.33	0.30	1000	0.99

As the survival data from the infected subset is divided into individuals that died close to post-infection treatment, or survived this initial time-period, the survival data were further analysed as two separate datasets to understand whether diet affected mortality in this two time points differently. Survival data was divided into flies that died before day 10 and after day 10 (10 days was picked as this was the first day when the number of dead flies was below two flies per day, see Appendix B, Figure S2.5). However, it should be noted that these datasets have very low sample sizes (many include no flies, some as low as 1-3 flies, see Appendix B, Table S2.3). Therefore, the results of these analyses should be interpreted with caution and only as preliminary analysis without further testing specifically for this effect with large enough sample sizes in each group.

Due to a lack of injured and unstressed control flies dying prior to 10 days post-infection treatment, an event history binomial model including only infected flies was used to analyse how mortality patterns changed across the two time points. This model suggests that mortality risk decreased 10 days after infection treatments compared to mortality in the first 10 days (Figure 2.3; Table 2.2; Post-10 days = -4.46 (95% CI = -5.87 to -3.24), p = <0.001). Increasing protein decreased mortality risk, however as there was a significant interaction between protein and post-10 days, for individuals dying after 10 days, the effect of protein significantly decreased in comparison to prior to 10 days (Figure 2.3; Table 2.2; Protein = -0.62 (95% CI = -1.10 to -0.18), p = 0.005; Protein:Post-10 days = 0.78 (95% CI = 0.29 to 1.28), p = <0.001). However, this pattern may be due to the sample size and the change in mortality patterns rather than a change in diet effects, as many flies from the low and high protein diets are not included in the post-10 days after infection treatments subset due to many flies dying before this cut off point.

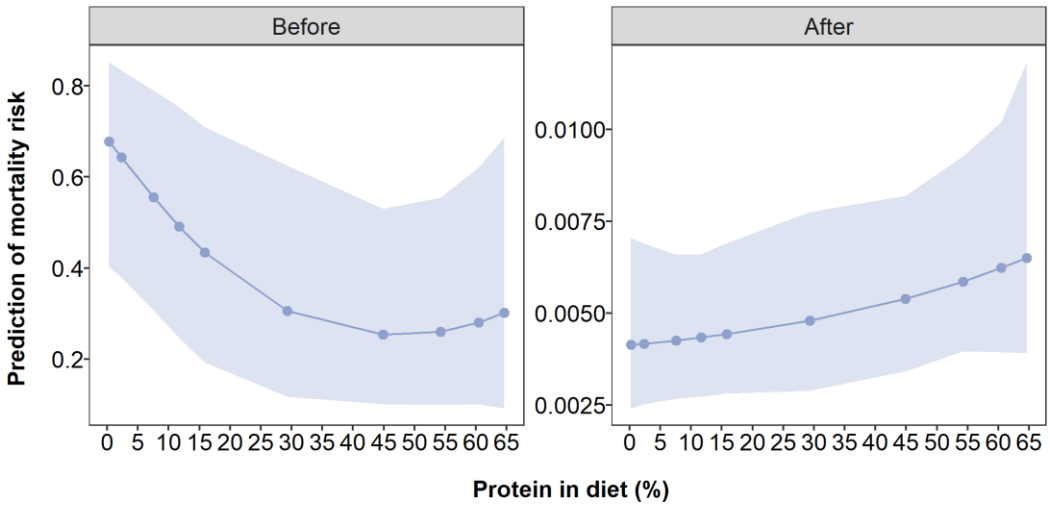

Figure 2.3: Model predictions from an event history binomial model for the effect of protein restriction on mortality risk per day of flies infected with a bacterial pathogen, for flies which died prior to ("Before") and after 10 days post-infection treatment ("After"). In the binomial model, for each day each fly was coded as 0 for alive and 1 for dead. Protein and protein^2 are mean centered to standard deviation of 1. Shaded areas are 95% credible intervals. Please note that the predictions are not curved as parabolas, as the model predictions have been transformed back to the data scale. The two time points have different y-axis scales for ease of interpretation.

Table 2.2: Model summary of effects of protein restriction on mortality risk per day from an event history binomial model including only infected flies and with mortality risk estimated for prior and post-10 days of infection treatment. In the binomial model, per each fly for each day, 0 coded for flies alive and 1 for dead. Protein and protein^2 are mean centered to standard deviation of 1. The model included random effects of Individual ID (posterior mean = 0.02 (95% credible interval (CI) = 9.81×10^{-9} to 0.08), effective sample size = 1778) and Experimental day (posterior mean = 2.80 (95% CI = 1.52 to 4.27), effective sample size = 755). Significant results below significance level $\alpha = 0.05$ are bolded.

	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
Intercept	-0.86	-2.05	0.48	1609	0.19
Protein	**-0.62**	**-1.10**	**-0.18**	**1610**	**0.005**
Protein^2	0.43	-0.13	1.07	2000	0.18
Post-10 days	**-4.46**	**-5.87**	**-3.24**	**1201**	**<0.001**
Protein:Post-10 days	**0.78**	**0.29**	**1.28**	**1609**	**<0.001**
Infection:Protein^2	-0.40	-1.05	0.29	2000	0.27

Mortality in the two time points was further analysed separately to understand whether these patterns differed from the other stress treatments. For flies that died prior to 10 days post-treatment, the effect of diet could only be analysed for the infected subset due to the lack of mortality of flies in the control and infection group. Therefore, these models do not test whether the diet patterns in the infected subset are significantly different to the control group. Due to poor model fitting, an event history binomial model could not be run for the infected flies that died prior to day 10 (data not shown). A model analysing lifespan suggests that for flies that died prior to 10 days post-infection treatment, increasing protein increased lifespan up to a point, and as the effect of protein is quadratic, this increase appeared to plateau at the highest protein diets (Figure 2.4; Table 2.3; Protein = 1.50 (95% CI = 0.74 to 2.38), p = 0.002; Protein^2 = -0.46 (95% CI = -0.86 to -0.04), p = 0.024). Although the assumption of proportional hazards was not met, a Cox Proportional hazards model shows qualitatively similar results (Appendix B, Figure S2.6; Table S2.4).

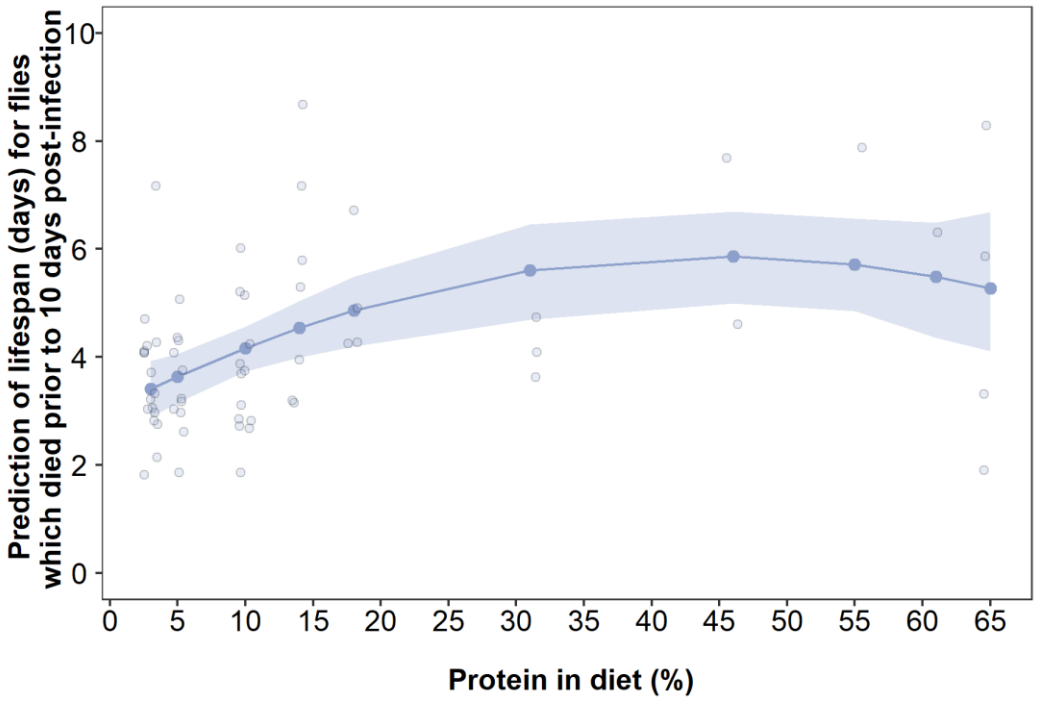

Figure 2.4: Model predictions from a linear model for the effect of protein restriction on lifespan of flies infected with a bacterial pathogen, for flies which died prior to 10 days post-infection. Protein and protein^2 are mean centered to standard deviation of 1. Shaded areas are 95% credible intervals. A secondary set of points show the lifespan data. Please note that the predictions are not curved as parabolas, as the model predictions have been transformed back to the data scale and the changing scale for the y-axis between the two time-points.

Table 2.3: Model summary of effects of protein restriction on lifespan from a linear model including only infected flies that died prior to 10 days post-infection treatment. Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded.

	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
Intercept	**4.65**	**4.03**	**5.16**	**1000**	**<0.001**
Protein	**1.50**	**0.74**	**2.38**	**1000**	**0.002**
Protein^2	**-0.46**	**-0.86**	**-0.04**	**1000**	**0.024**

For flies that survived at least 10 days post-stress treatment, mortality patterns for control and injured flies were similar to the full model including all individuals (Figure 2.1 & 2.5, Table 2.1 & 2.4), most likely as not many of these flies died prior to day 10 (Appendix B, Table S2.3). Similar to the full model, infected flies had higher mortality than the control flies (Figure 2.1 & 2.5, Table 2.1 & 2.4, Infection = 0.72 (95% CI = 0.26 to 1.16), p = <0.001). However, in comparison to the full model, in the model only including flies that died post 10-days after stress treatments, infection treatment has no significant interaction with protein, and the quadratic relationship between diet and mortality for infected flies was more linear compared to the control flies (Figure 2.5, Table 2.4; Infection:Protein = 0.20 (-0.06 to 0.46), p = 0.16; Protein^2 = 0.51 (0.25 to 0.76), p = <0.001; Infection:Protein^2 = -0.50 (-0.88 to -0.09), p = 0.01). These patterns may suggest that protein has a different effect on mortality on flies that survive the initial higher mortality post-infection treatment. However, as can be seen from the wide 95% credible intervals associated with the infected flies, as the low and high protein diets only contained a few flies that survived the initial 10 days, the remaining flies may not be representative of general patterns as they may have more comparable survival to the control group due to not being infected. Furthermore, the analysis lacks the sample sizes to have robust statistical testing of these effects. Analysing the lifespan data or the survival data with a Cox proportional hazard model (with assumption of proportional hazards not met), qualitatively similar results were found (Appendix B, Figure S2.7 & S2.8; Table S2.5 & S2.6).

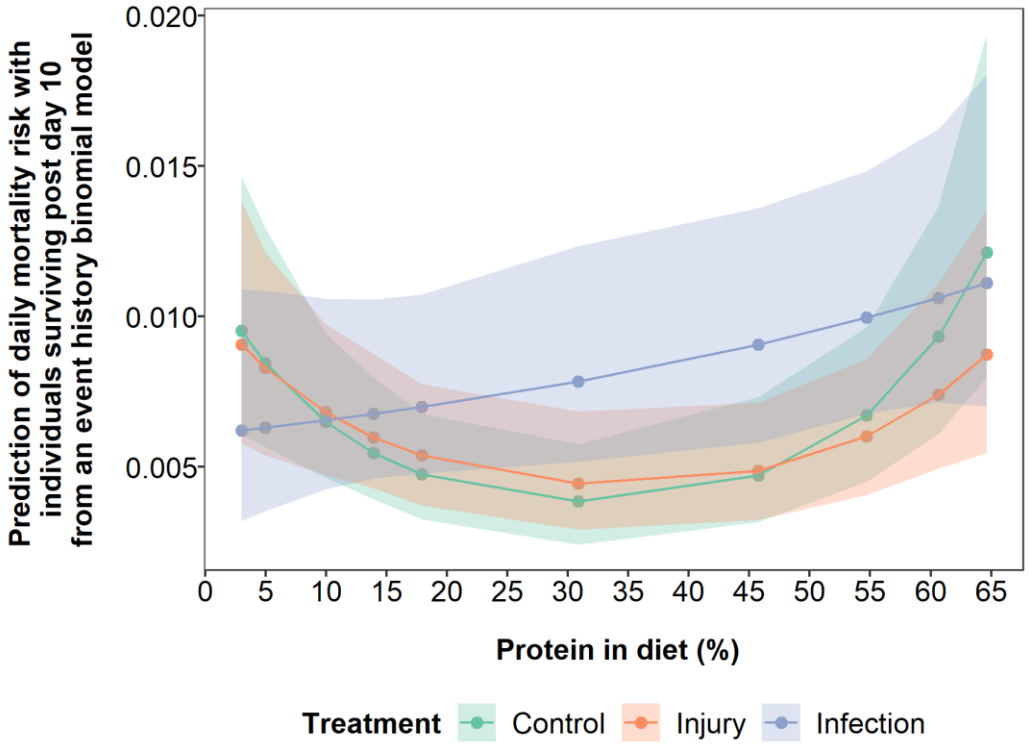

Figure 2.5: Model predictions from an event history binomial model for the effect of protein restriction on mortality risk per day of flies infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines) for flies that survived at least 10 days post-stress treatments. In the binomial model, for each day each fly was coded as 0 for alive and 1 for dead. Protein and protein2 are mean centered to standard deviation of 1. Shaded areas are 95% credible intervals. Please note that the predictions are not curved as parabolas, as the model predictions have been transformed back to the data scale.

Table 2.4: Model summary of effects of protein restriction and stress treatments on mortality risk per day from an event history binomial model for flies which survived at least 10 days post-stress treatment. In the binomial model, per each fly for each day, 0 coded for flies alive and 1 for dead. Protein and protein^2 are mean centered to standard deviation of 1. The model included random effects of Individual ID (posterior mean = 0.01 (95% credible interval (CI) = 4.27 x 10^{-9} to 0.04), effective sample size = 1000) and Experimental day (posterior mean = 2.96 (95% CI = 2.01 to 3.92), effective sample size = 723.9). Significant results below significance level $\alpha = 0.05$ are bolded.

	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
Intercept	**-5.55**	**-5.98**	**-5.12**	**1000**	**<0.001**
Injury treatment	0.13	-0.31	0.53	1000	0.55
Infection treatment	**0.72**	**0.26**	**1.16**	**1000**	**<0.001**
Protein	0.01	-0.14	0.16	1124	0.93
Protein^2	**0.51**	**0.25**	**0.76**	**1000**	**<0.001**
Injury:Protein	-0.07	-0.30	0.15	1105	0.51
Infection:Protein	0.20	-0.06	0.46	1000	0.16
Injury:Protein^2	-0.15	-0.47	0.21	1000	0.41
Infection:Protein^2	**-0.50**	**-0.88**	**-0.09**	**1000**	**0.01**

2.4.3 Reproduction:

Stress treatment had no significant effect on the lifetime number of eggs produced at mean levels of dietary protein (Figure 2.6 & 2.7; Table 2.5; Injury = 0.19 (95% CI = -0.34 to 0.72), p = 0.49; Infection = -0.33 (95% CI = -0.90 to 0.16), p = 0.26), but there was a significant interaction between stress treatment and both protein and its squared term (Table 2.5; Infection:Protein = 0.47 (95% CI = 0.16 to 0.77), p = 0.01; Infection:Protein^2 = -0.47 (95% CI = -0.93 to -0.04), p = 0.04). For the baseline of control unstressed flies, lifetime egg production was highest at high but not the highest protein levels, with flies on low protein diets in particular producing very few eggs (Figure 2.6 & 2.7; Table 2.5; Protein = 1.45 (95% CI = 1.23 to 1.64), p = <0.001; Protein^2 = -1.36 (95% CI = -1.68 to -1.02), p = <0.001). Therefore, the protein and stress treatment interactions suggest that infected individuals had a higher linear increase in lifetime eggs with increasing protein, but this relationship was also more curved, than in either the control or injury group. Despite these significant interactions, the broad pattern of change in egg counts with changing protein level is similar across stress treatments (Figure 2.6 & 2.7).

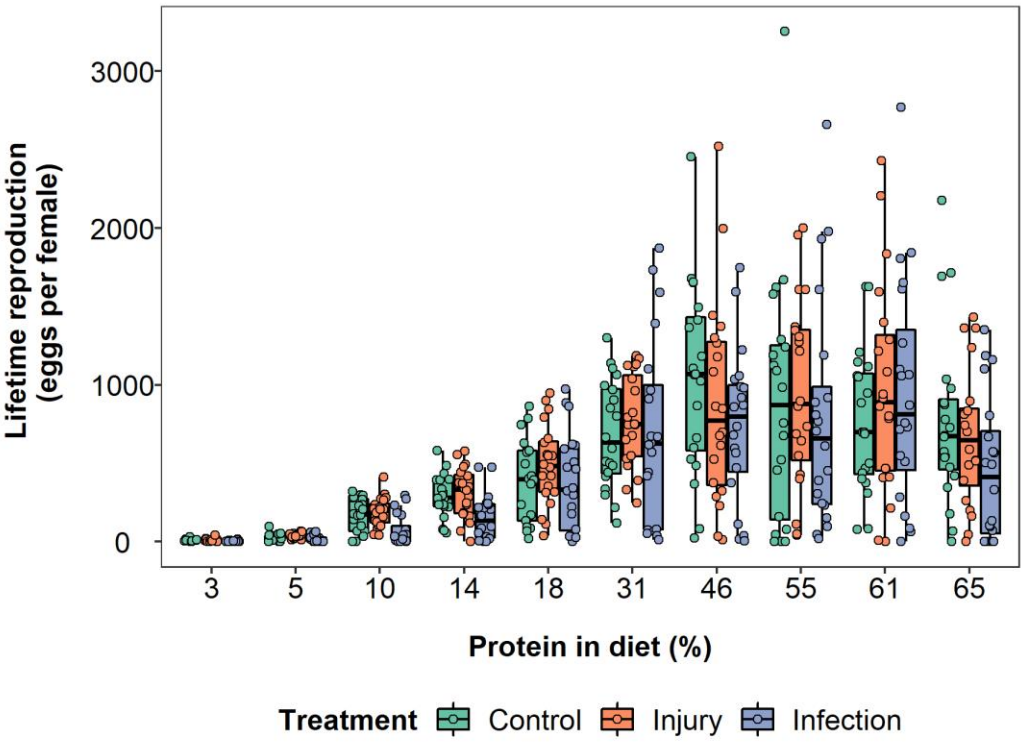

Figure 2.6: Effect of protein restriction on the lifetime egg production of flies infected with a bacterial pathogen (blue lines and data points), injured by a pinprick (orange lines and data points) or with no treatment (green lines and data points). The lines in the box plots indicate median lifespan (50% quantile), the boxes are the interquartile range (25% to 75% quantiles) and the whiskers are minimum or maximum quartiles (25% - 1.5 x interquartile range, 75% + 1.5 x interquartile range).

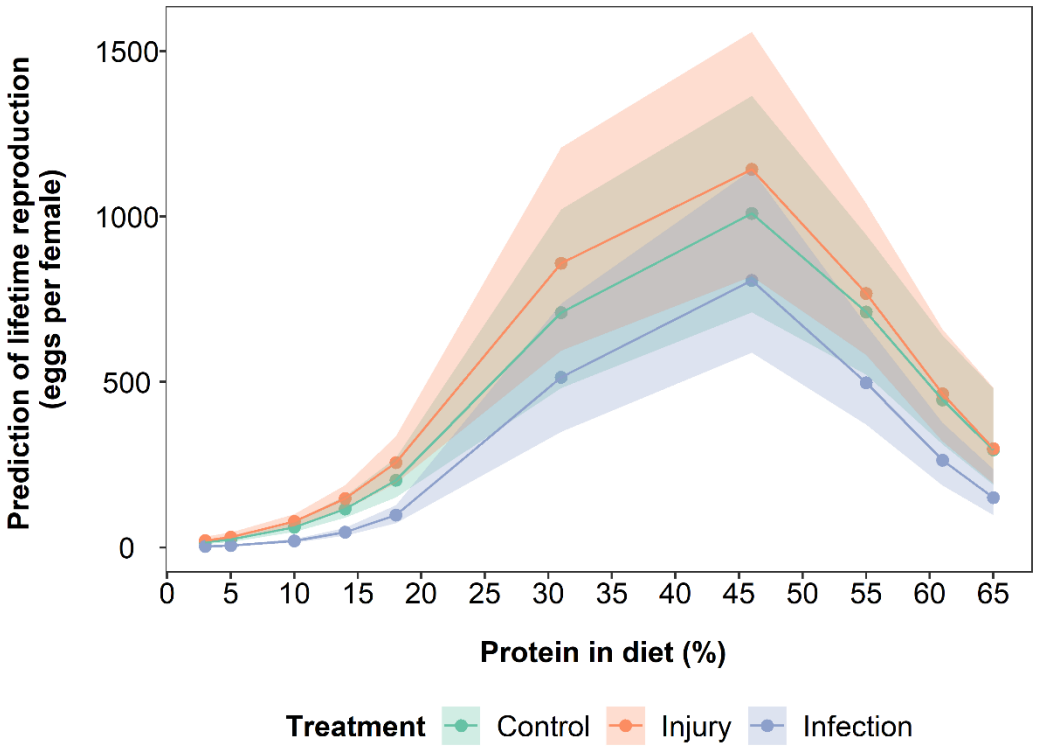

Figure 2.7: Model predictions of the effect of protein restriction on the lifetime egg production of flies infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines). Shaded areas are 95% credible intervals. Protein and protein^2 are mean centered to standard deviation of 1. Please note that the predictions are not curved as parabolas, as the model predictions have been transformed back to the data scale.

Table 2.5: Model summary of effects of protein restriction and stress treatments on lifetime eggs produced. Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded.

	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
Intercept	**6.55**	**6.17**	**6.92**	**1205**	**<0.001**
Injury treatment	0.19	-0.34	0.72	1000	0.49
Infection treatment	-0.33	-0.90	0.16	1330	0.264
Protein	**1.45**	**1.23**	**1.64**	**1000**	**<0.001**
Protein^2	**-1.36**	**-1.68**	**-1.02**	**1000**	**<0.001**
Injury:Protein	-0.09	-0.39	-1.02	1000	0.60
Infection:Protein	**0.47**	**0.16**	**0.77**	**1000**	**0.01**
Injury:Protein^2	-0.02	-0.45	0.46	1000	0.93
Infection:Protein^2	**-0.47**	**-0.93**	**-0.04**	**1000**	**0.04**

To control for variation in lifetime egg production due to differences in lifespan, early-life egg production was also analysed. For eggs produced in the first week, excluding the first day, the patterns were similar to those of lifetime egg production (Figure 2.7 & 2.9; Table 2.5 & 2.6). However, there was no interaction between stress treatment and protein on early-life egg production (Figure 2.9; Table 2.6; Infection:Protein = -0.24 (95% CI = -0.74 to 0.20), p = 0.32; Infection:Protein^2 = -0.14 (95% CI = -0.85 to 0.57), p = 0.74). The decline in egg production at higher protein levels was reduced compared to lifetime egg production, such that early-life egg production plateaus after reaching a maximum at intermediate protein levels, with a slight decline at very high protein levels (Figure 2.9; Table 2.6; Protein^2 = -0.86 (95% CI = -1.34 to -0.41), p = <0.001). Similar patterns were seen in models of lifetime egg production with mean centred lifespan included in the model (Appendix B, Figure S2.9; Table S2.6), suggesting that differences in lifetime reproduction between stress treatments are driven by the short lifespan of infected flies on low protein diets. As might be expected, flies

with longer lifespans had more eggs over their life than shorter-lived flies (Appendix B, Table S2.7, Lifespan = 0.93 (95% CI = 0.83 to 1.04), p = <0.001).

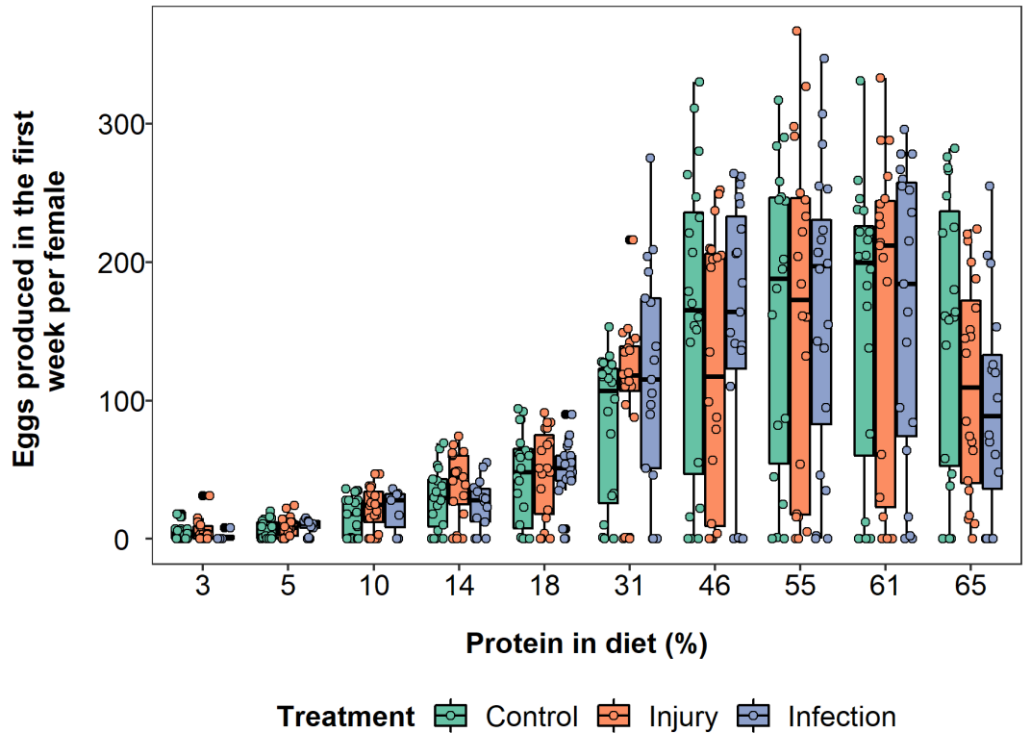

Figure 2.8: Effect of protein restriction on the early-life egg production of flies infected with a bacterial pathogen (blue lines and data points), injured by a pinprick (orange lines and data points) or with no treatment (green lines and data points). Early-egg production consists of the first seven days of egg production without the first day (see methods). The lines in the box plots indicate median number of eggs produced (50% quantile), boxes are the interquartile range (25% to 75% quantiles) and whiskers are minimum or maximum quartiles (25% - 1.5 x interquartile range, 75% + 1.5 x interquartile range).

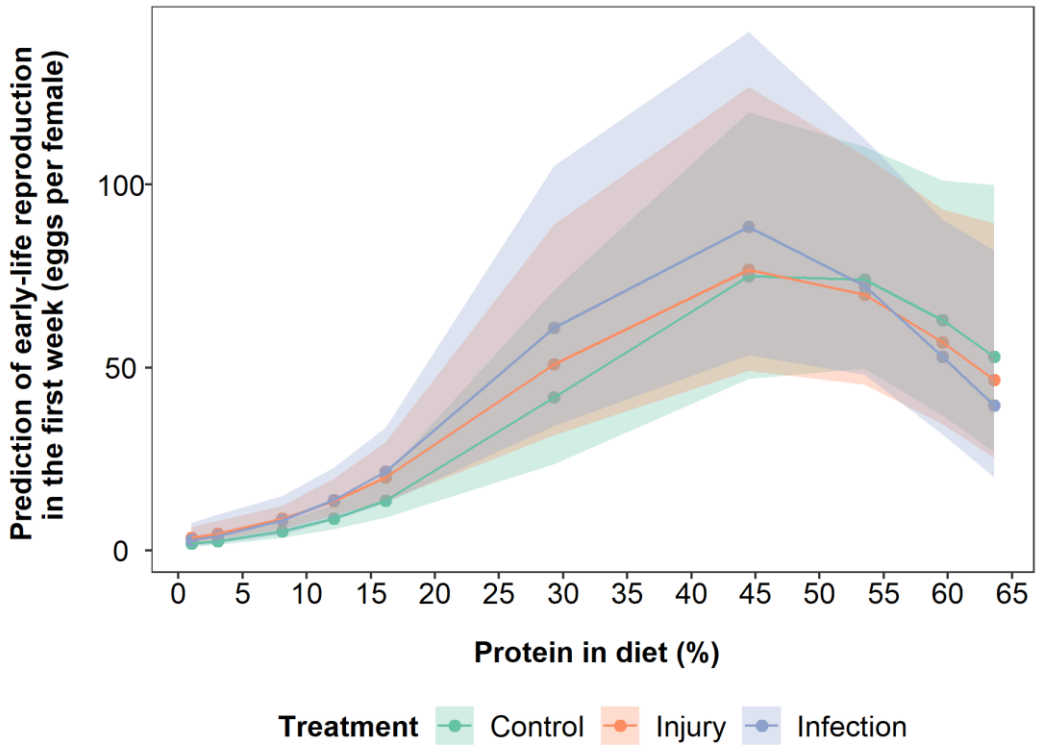

Figure 2.9: Model predictions of the effect of protein restriction on the early-life egg production of flies infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines). Early-life egg production consists of the first seven days of egg production without the first day (see methods). Shaded areas are 95% 95% credible intervals. Protein and protein^2 are mean centered to standard deviation of 1. Please note that the predictions are not curved as parabolas, as the model predictions have been transformed back to the data scale.

Table 2.6: Model summary of effect of protein restriction and stress treatment on early-life egg production (first week discounting the first day, see methods). Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded.

	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
Intercept	**3.82**	**3.30**	**4.41**	**1156**	**<0.001**
Injury treatment	0.18	-0.56	0.92	1000	0.69
Infection treatment	0.36	-0.37	1.16	1330	0.37
Protein	**1.34**	**1.06**	**1.64**	**1000**	**<0.001**
Protein^2	**-0.86**	**-1.34**	**-0.41**	**1149**	**<0.001**
Injury:Protein	-0.29	-0.69	0.10	1000	0.17
Infection:Protein	-0.24	-0.74	0.20	1000	0.32
Injury:Protein^2	0.05	-0.60	0.66	1000	0.86
Infection:Protein^2	-0.14	-0.85	0.57	1198	0.74

2.4.4 Ageing:

2.4.4.1 Daily egg production:

There were numerous significant two- and three-way interactions in the daily egg production model. Control unstressed individuals on average protein diets produced most eggs per day early in life, with significantly declining egg production with age (Figure 2.10 & 2.11; Table 2.7; Age = -0.32 (95% CI = -0.40 to -0.23), n = <0.001), but this decline was non-linear (Figure 2.10 & 2.11; Table 2.7; Age^2 = -0.52 (95% CI = -0.59 to -0.44), p = <0.001). With higher protein, control individuals were able to produce significantly more eggs per day (Figure 2.10 & 2.11; Table 2.7; Protein = 1.31 (95% CI = 1.12 to 1.52), p = <0.001). However, at very low and high levels of protein, egg production reduced (Figure 2.10 & 2.11; Table 2.7; $Protein^2$ = -1.5 (95% CI = -1.51 to -1.81), p = <0.001). For these control unstressed individuals, the decline in reproduction with age is steepest and less curved at higher protein levels, but not at the highest protein levels (Figure 2.10 & 2.11; Table 2.7; $Protein^2$:Age = -0.24 (95% CI = -0.32 to -0.16), p = <0.001; $Protein^2$:Age^2 = 0.30 (95% CI = 0.22 to 0.38), p = <0.001).

For infected individuals, the three-way interactions suggest that the curved relationship between reproduction and age is greatest for individuals on intermediate to high (but not the highest) protein diets in comparison to the control flies (Figure 2.10 & 2.11; Table 2.7; Infection:$Protein^2$:Age = 0.22 (95% CI = 0.09 to 0.35), p = 0.005; Infection:$Protein^2$:Age^2 = -0.37 (95% CI = -0.51 to -0.23) p = <0.001). Injured individuals show similar patterns to infected individuals, but in terms of the curvature with age, this change compared to the control flies is generally less than for infected individuals (Figure 2.10 & 2.11; Table 2.7; Injury:$Protein^2$:Age = -0.12 (95% CI = -0.23 to -0.23), p = 0.04; Injury:$Protein^2$:Age^2 = -0.13 (95% CI = -0.25 to -0.02), p = 0.03). There was a significant effect of lifespan on daily egg production, suggesting that longer-lived individuals produced more eggs per day (Table 2.7; Lifespan = 0.21 (95% CI = 0.11 to 0.31), p = <0.001).

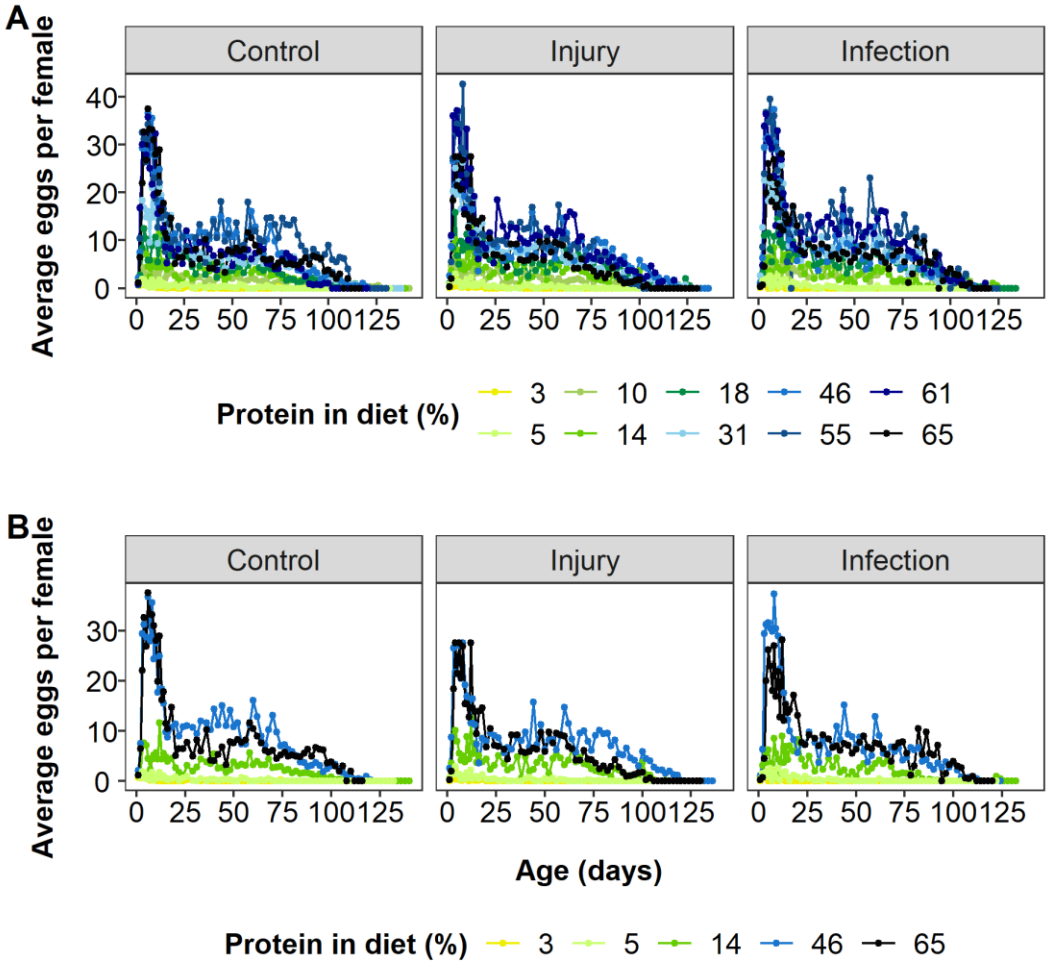

Figure 2.10: The pattern of ageing in egg production for each protein restriction diet for flies infected with a bacterial pathogen ("Infection"), injured by a pinprick ("Injury") or with no treatment ("Control"). The average eggs laid per day across all flies per diet and stress treatment is plotted and the associated errors have been removed for clarity. (A) All diets for each stress treatment; (B) A subset of protein restriction diets to illustrate the effects of protein restriction with low (yellow line) intermediate (blue line) and high protein content (black line).

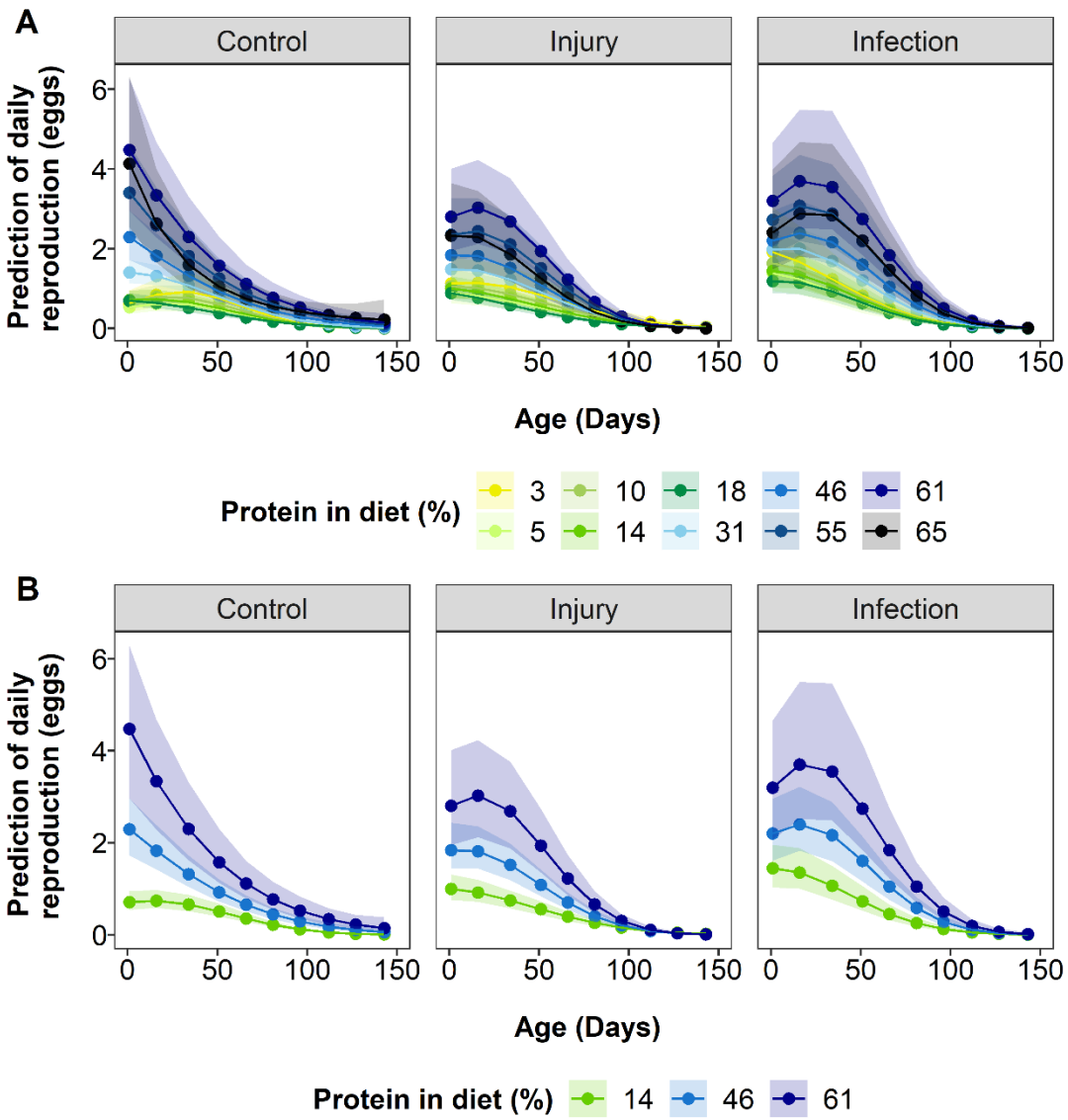

Figure 2.11: Model predictions of the effect of protein restriction and age on daily egg production of flies infected with a bacterial pathogen ("Infection"), injured by pinprick ("Injury") or with no treatment ("Control"). Model predictions are shown for (A) all diets, or (B) for ease of interpretation, for a subset of protein restriction diets to illustrate the effects of protein restriction with low (green line), intermediate (light blue line) and higher protein content (dark blue line). Shaded areas are 95% credible intervals. Protein, protein^2 and lifespan are mean centered to standard deviation of 1. Please note that the predictions are not curved as parabolas, as the model predictions have been transformed back to the data scale.

Table 2.7: Model summary of effects of protein restriction, age and stress treatment for daily egg production on flies. Protein, protein^2, age, age^2 and lifespan are mean centered to standard deviation of 1. The model included random effects of Individual ID (posterior mean = 1.76 (95% CI = 1.53 to 2.01), effective sample size = 1334). Lifespan (mean centered) is included to account for selective disappearance. Significant results below significance level $\alpha = 0.05$ are bolded.

	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
Intercept	**1.61**	**1.23**	**1.98**	**1334**	**<0.001**
Injury treatment	0.19	-0.35	0.68	1193	0.48
Infection treatment	-0.11	-0.66	0.47	1334	0.72
Protein	**1.31**	**1.12**	**1.52**	**1334**	**<0.001**
Protein^2	**-1.51**	**-1.81**	**-1.19**	**1334**	**<0.001**
Age	**-0.32**	**-0.40**	**-0.23**	**1222**	**<0.001**
Age^2	**-0.52**	**-0.59**	**-0.44**	**1334**	**<0.001**
Lifespan	**0.21**	**0.11**	**0.31**	**1334**	**<0.001**
Injury:Protein	0.08	-0.22	0.35	1334	0.58
Infection:Protein	-0.21	-0.54	0.11	1334	0.22
Injury:Protein^2	-0.05	-0.51	0.36	1477	0.82
Infection:Protein^2	**0.51**	**0.04**	**0.97**	**1334**	**0.03**
Injury:Age	**0.13**	**0.01**	**0.24**	**1334**	**0.03**
Infection:Age	**-0.29**	**-0.42**	**-0.14**	**1334**	**<0.001**
Injury:Age^2	0.08	-0.03	0.19	1063	0.15
Infection:Age^2	**0.27**	**0.13**	**0.41**	**1334**	**0.002**
Protein:Age	0.02	-0.04	0.08	1334	0.56
Protein:Age^2	-0.04	-0.09	0.02	1660	0.22
Protein^2:Age	**-0.24**	**-0.32**	**-0.16**	**1334**	**<0.001**
Protein^2:Age^2	**0.30**	**0.22**	**0.38**	**1116**	**<0.001**
Injury:Protein:Age	**0.14**	**0.05**	**0.22**	**1334**	**0.002**

Infection:Protein:Age	**0.11**	**0.001**	**0.20**	**1334**	**0.04**
Injury:Protein:Age²	**-0.14**	**-0.22**	**-0.06**	**1334**	**<0.001**
Infection:Protein:Age²	0.02	-0.09	0.13	1334	0.64
Injury:Protein²:Age	**-0.12**	**-0.23**	**-0.005**	**1360**	**0.04**
Infection:Protein²:Age	**0.22**	**0.09**	**0.35**	**1334**	**0.005**
Injury:Protein²:Age²	**-0.13**	**-0.25**	**-0.02**	**1175**	**0.03**
Infection:Protein²:Age²	**-0.37**	**-0.51**	**-0.23**	**1334**	**<0.001**

2.4.4.2 Gut deterioration (smurf) assay:

To assess gut integrity as a measure of ageing, flies were fed blue food and were scored as a smurf if they turned blue due to the blue food leaking from the gut. Only 11.0% of flies (63/573, excluding censored flies) became smurfs throughout the experiment, where the first smurf started appearing from day 18, so these results should be interpreted with some caution. With individuals that died prior to day 18 removed from the analysis, there was a significant two-way interaction between injury treatment and protein content, where the decline in the proportion of smurfs with increasing protein content was stronger in the injury treatment than in the control treatment (Figure 2.12 & 2.13; Table 2.8; Protein = -0.78 (95% CI = -1.32 to -0.23), p = <0.001; Injury:Protein = -1.94 (95% CI = -3.92 to -0.19), p = 0.008). There was also a significant interaction between injury treatment and the quadratic effect of protein (Table 2.8; Injury:Protein² = -2.10 (95% CI = -3.96 to -0.37), p = 0.01). This suggests that in injured individuals, the proportion of smurfs peaked at more intermediate protein levels and then declined at both high and the lowest protein levels compared to the control flies. As smurfs start appearing at a later-life stage, low survival in the different diet and stress treatment combinations to day 18 and later in the experiment may be driving this effect.

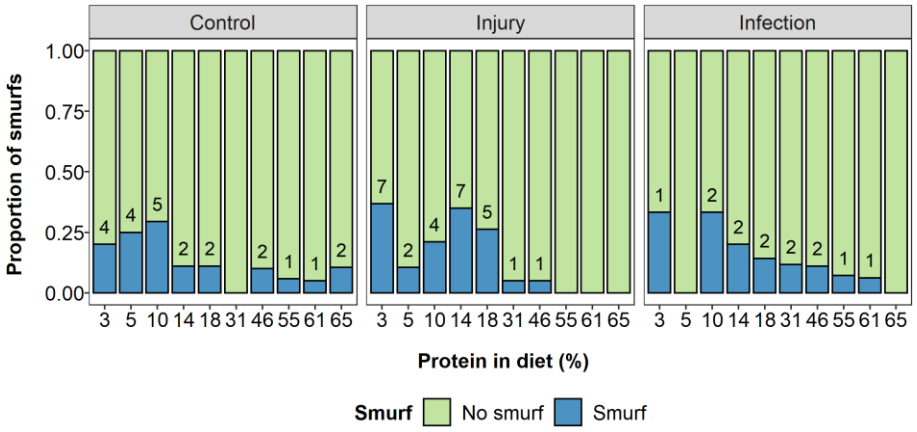

Figure 2.12: Effects of protein restriction on proportion of smurfs (blue bars) or no smurfs (green bars) across life of flies infected with a bacterial pathogen ("Infection", number of smurfs = 23), injured by a pinprick ("Injury", number of smurfs = 25) or with no treatment ("Control", number of smurfs = 15). Numbers indicate the number of smurfs in the diet and stress treatment combination.

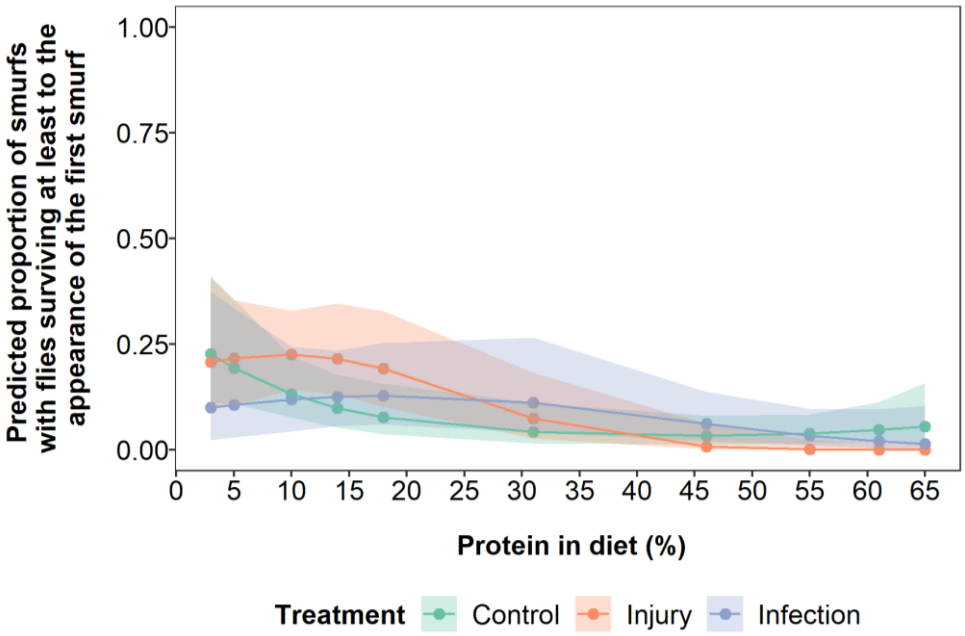

Figure 2.13: Model predictions of the effect of protein restriction on the proportion of flies developing into a smurf of flies infected with a bacterial pathogen (blue data points and lines), injured by pinprick (orange data points and lines) or with no treatment (green data points and lines). Only individuals that survived at least 18 days are included (first appearance of a smurf phenotype). Protein and protein^2 are mean centered to standard deviation of 1. Shaded areas are 95% credible intervals.

Table 2.8: Model summary of effects of protein restriction and stress treatment on proportion of flies developing into a smurf. Only individuals that survived at least 18 days are included (first appearance of a smurf phenotype). Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded.

	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
Intercept	**-3.13**	**-4.19**	**-2.08**	**1000**	**<0.001**
Injury treatment	0.63	-0.94	2.06	1106	0.41
Infection treatment	1.06	-0.50	2.52	1176	0.17
Protein	**-0.78**	**-1.32**	**-0.23**	**1000**	**<0.001**
Protein^2	0.65	-0.18	1.46	1000	0.15
Injury:Protein	**-1.94**	**-3.92**	**-0.19**	**1000**	**0.008**
Infection:Protein	0.17	-0.81	1.19	1000	0.74
Injury:Protein^2	**-2.10**	**-3.96**	**-0.37**	**1000**	**0.01**
Infection:Protein^2	-1.23	-2.75	0.16	1000	0.09

2.4.4.3 Negative geotaxis (NG) assay:

By assessing escape response as a measure of ageing, there were no differences between control, injured or infected flies in passing the test (Figure 2.14 & 2.15; Table 2.9). Having controlled for lifespan, the proportion of flies passing the NG test declined more steeply with age on higher protein diets (Figure 2.14 & 2.15, Table 2.9, Protein:Age = -0.78 (95% CI = -1.06 to -0.49), p = <0.001). The likelihood of passing the test decreased with increasing protein (Table 2.9; Protein = -0.65 (95% CI = -1.01 to -0.32), p = <0.001), but the rate of this decline slowed at the highest protein levels (Table 2.9; Protein^2 = -0.70 (95% CI = -1.21 to -0.21), p = 0.01). Older flies were less likely to pass the test (Table 2.9; Age = -3.57 (95% CI = -4.04 to -3.07), p = <0.001). There was an effect of selective disappearance, where longer-lived individuals passed the test at a higher rate than individuals with shorter lifespans did (Table 2.9, Lifespan = 0.84 (95% CI = 0.64 to 1.02), p = <0.001).

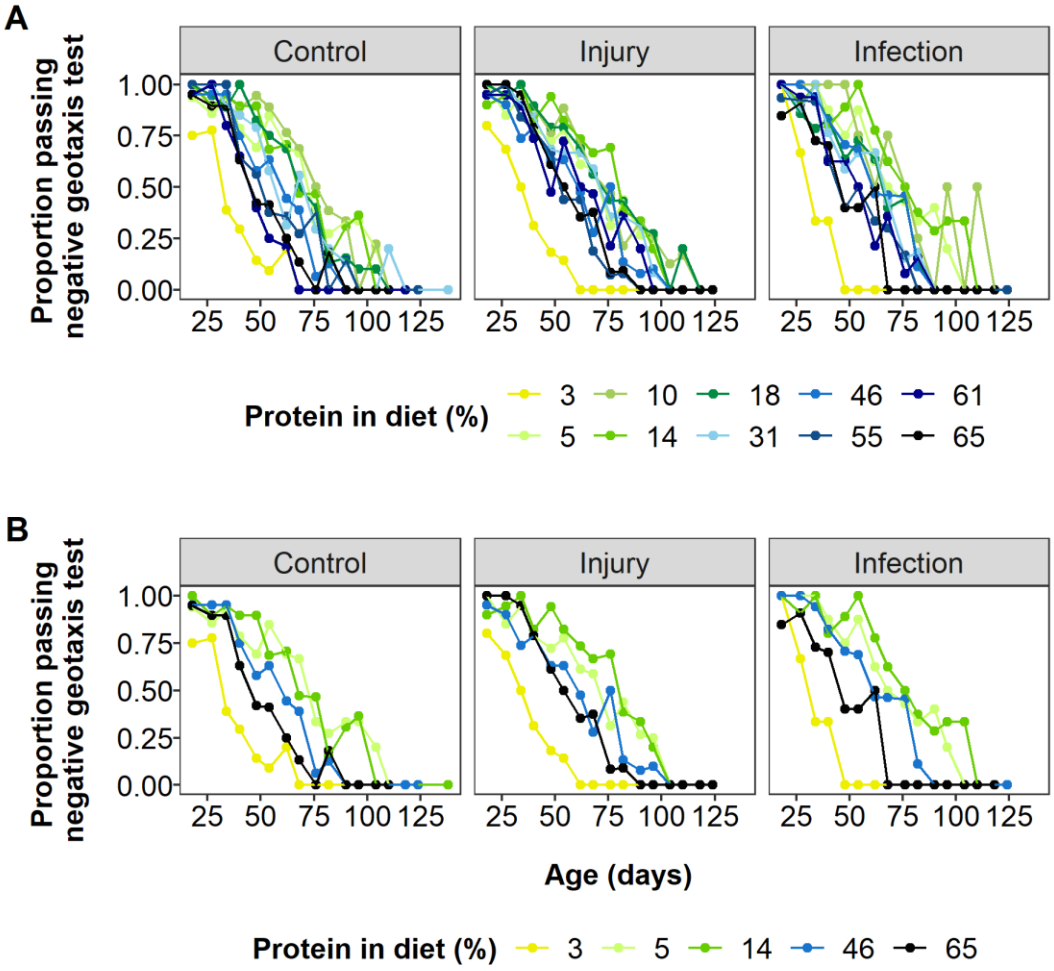

Figure 2.14: Effects of protein restriction on the proportion of flies passing the negative geotaxis test under 60 seconds per week of flies infected with a bacterial pathogen ("Infection"), injured by a pinprick ("Injury"), or with no treatment ("Control") (A). For ease of interpretation, a subset of diets is shown in (B) to illustrate the effects of protein restriction with low (yellow line) intermediate (pale blue line) and high protein content (black line).

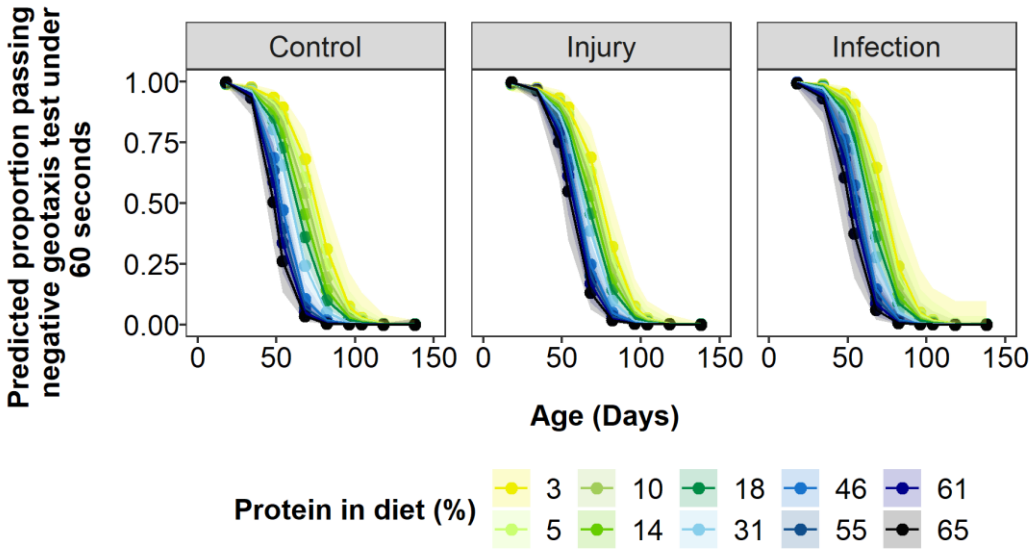

Figure 2.15: Model predictions of the effect of protein restriction and age on proportion passing negative geotaxis test under 60 seconds per week with flies infected with a bacterial pathogen ("Infection"), injured by pinprick ("Injury") or with no treatment ("Control"). Shaded areas are 95% credible intervals. Protein, protein^2 and lifespan are mean centered to standard deviation of 1.

Table 2.9: Model summary of effects of protein restriction, age and stress treatment for passing negative geotaxis test under 60 seconds. Protein, protein^2, age, age^2 and lifespan are mean centered to standard deviation of 1. Lifespan (mean centered) is included to account for selective disappearance. The model included the random effect of Individual ID (posterior mean = 3.03 (95% CI = 2.35 to 3.73), effective sample size = 1000). Significant results below significance level α = 0.05 are bolded.

	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
Intercept	**1.01**	**0.39**	**1.63**	**892.4**	**0.002**
Injury treatment	0.52	-0.38	1.37	1000	0.23
Infection treatment	0.35	-0.59	1.32	1000	0.49
Protein	**-0.65**	**-1.01**	**-0.32**	**1000**	**<0.001**
Protein^2	**-0.70**	**-1.21**	**-0.21**	**1060**	**0.01**
Age	**-3.57**	**-4.04**	**-3.07**	**1000**	**<0.001**
Age^2	-0.13	-0.61	0.36	902.6	0.58
Lifespan	**0.84**	**0.64**	**1.02**	**1000**	**<0.001**
Injury:Protein	0.38	-0.07	0.86	1197.5	0.12
Infection:Protein	0.20	-0.40	0.81	1000	0.53
Injury:Protein^2	0.10	-0.60	0.78	1102.8	0.78
Infection:Protein^2	-0.04	-0.76	0.89	1101.4	0.93
Injury:Age	0.48	-0.15	1.17	1098.6	0.17
Infection:Age	-0.17	-1.03	0.51	1039.8	0.69
Injury:Age^2	-0.30	-0.86	0.43	1000	0.37
Infection:Age^2	-0.26	-1.12	0.47	1000	0.51
Protein:Age	**-0.78**	**-1.06**	**-0.49**	**1108.6**	**<0.001**
Protein:Age^2	0.16	-0.13	0.42	1000	0.27
Protein^2:Age	0.06	-0.33	0.48	1124.5	0.79
Protein^2:Age^2	0.15	-0.28	0.50	1000	0.45

Injury:Protein:Age	0.28	-0.11	0.63	1000	0.16
Infection:Protein:Age	0.37	-0.10	0.91	1132.2	0.17
Injury:Protein:Age^2	-0.17	-0.54	0.19	1045.3	0.40
Infection:Protein:Age^2	-0.35	-0.83	0.10	1227	0.14
Injury:Protein^2:Age	-0.17	-0.72	0.39	1000	0.54
Infection:Protein^2:Age	-0.03	-0.63	0.75	1000	0.95
Injury:Protein^2:Age^2	0.09	-0.45	0.60	1000	0.73
Infection:Protein^2:Age^2	0.13	-0.56	0.80	1000	0.69

2.5 Discussion:

Our results provide a rare test of the predictions of two alternative evolutionary explanations for the commonly observed extension of lifespan in response to dietary restriction (DR). The nutrient recycling hypothesis (NRH) predicts that DR will not extend lifespan with the addition of injury and infection to the usually benign laboratory environment (Adler & Bonduriansky, 2014). Alternatively, the resource reallocation hypothesis (RRH) does not make this prediction (Shanley & Kirkwood, 2000). We applied two stressors and diets ranging in protein to carbohydrate (P:C) ratios to a population of outbred female *Drosophila melanogaster* to test these predictions. Our data showed that lifespan extension and delayed ageing with DR remained even with the addition of injury and infection, therefore supporting the RRH. In particular, survival and lifespan (for the full dataset) were maximised at intermediate protein levels and declined at very high and low protein levels across all stress treatments, typical of the DR response through P:C ratios (Carey et al., 2008; Skorupa et al., 2008; Lee, 2015) or through other methods of DR (e.g. Bishop & Guarente, 2007; Clancy et al., 2002; K P Lee et al., 2006; Magwere et al., 2004; Pletcher et al., 2005, see also meta-analysis Nakagawa et al., 2012). It should be noted that our results reflect broad changes in protein through changes in yeast:sugar, as in many other studies in *D. melanogaster* (e.g. Lee et al., 2008; Skorupa et al., 2008; Bruce et al., 2013). Therefore these effects may be a direct result of changes in micronutrients or specific amino acids (e.g. Simpson et al., 2015; Piper et al., 2017; Zanco et al., 2020).

A small number of other studies have also considered predictions from the NRH using alternative approaches to the ones used here. One tested the prediction that reproduction should decline if autophagy is inhibited under DR, but found that this was not the case in *Caenorhabditis elegans* (Travers et al., 2020). An experimental evolution study in *D. melanogaster* males hypothesised that according to the NRH, individuals under DR should be more efficient at using the available resources, and thus under long-term DR, experimental evolution lines

should evolve to have higher reproductive performance and increased survival with DR (Zajitschek et al., 2016). Against their predictions, there was no change in survival, although the DR selection lines did have higher reproductive performance (Zajitschek et al., 2016). A recent study using wild and captive antler flies found that protein restriction lowered mortality rate even in non-laboratory conditions (Mautz et al., 2019), contradicting the suggestion of the NRH that DR would have no benefit in the wild due to higher extrinsic mortality rate and stressors (Adler & Bonduriansky, 2014). This pattern was only present in one of the two years included in the study, highlighting the need for further studies. In general, it appears that the predictions of the NRH are not being met in the studies conducted to date (Adler & Bonduriansky, 2014).

Although the pattern of a tent-shaped response of survival and lifespan to increasing levels of protein restriction seen here is typical of many other studies (Carey et al., 2008; Skorupa et al., 2008; Lee, 2015; Jang & Lee, 2018; Kim et al., 2020), it does contrast with recent studies suggesting lifespan is maximised on diets with very low P:C (Lee et al., 2008; Maklakov et al., 2008; Fanson et al., 2009, 2012; Harrison et al., 2014; Solon-Biet et al., 2014; Jensen et al., 2015). These studies use a nutritional geometry approach where diets that vary in both calories and macronutrient ratio are used to separate the effects of these two variables. One reason our results may differ is the difference in the delivery of the diets, as most nutritional geometry studies in *D. melanogaster* have used liquid diets that allow fine scale measures of intake, but result in very low survival rates across all diets (Lee et al., 2008; Jensen et al., 2015). Studies using solid diets with *D. melanogaster* have found greater lifespans than in the liquid diet results, and have often found that lifespan was not maximised at the lowest protein diets (Skorupa et al. 2008; Bruce et al. 2013; Jang and Lee 2018; Kim et al. 2020, but see results for males in Kim et al. 2020). This suggests that diet delivery may have effects on survival, at least in *D. melanogaster* (see Maklakov et al. 2008; Fanson et al. 2009, 2012; Harrison et al. 2014; Solon-Biet et al. 2014 for other species and work

involving solid diets). More work is needed to understand the causes of the differences in lifespan between studies.

An alternative consideration that may explain why we did not see highest lifespans at the lowest protein diets might be due to the fact that the stock diet in our laboratory is a 14% protein diet, which is a relatively low protein diet. In an experimental evolution study in *D. melanogaster*, females from low protein experimental selection lines no longer had increased lifespan with protein restriction in comparison to females from control selection lines (Zajitschek et al., 2019). In our experiment, the outcrossed DGRP population had no increased lifespan with diets lower in protein than the 14% protein (1:6 P:C). This suggests that lifespan being maximised at intermediate rather than the lowest protein diets (as seen in some nutritional geometry studies above) may be reflective of previous dietary maintenance conditions.

Although survival was maximised at intermediate protein levels across all stress treatments, the survival of infected individuals on very low protein diets was particularly poor. A positive relationship between dietary protein content and survival when exposed to infection is a common finding (Table 2.1). This suggests that in general dietary protein is important for infection responses (e.g. Lee et al., 2006; Le Rohellec & Le Bourg, 2009; Cotter et al., 2019). However, there are some exceptions to the pattern (Table 2.1). Other than several methodological differences between studies (Table 2.1, Lee et al. 2017; Miller and Cotter 2018; Dinh et al. 2019; Sieksmeyer et al. 2019; Ponton et al. 2020; Roberts and Longdon 2020), these differences may be driven by the particular host-pathogen pair, as diet alters various components of the host response and pathogen performance, and these relationships vary between systems (e.g. Lee et al., 2006; Povey et al., 2009, 2014; Miller & Cotter, 2018; Cotter et al., 2019; Wilson et al., 2020). Further evidence for host-pathogen specific effects of diet comes from a meta-analysis of the effect of host nutrition on pathogen virulence, which found both positive or negative effects on virulence depending on the system (Pike et al., 2019). To understand the relationship between dietary protein and the response to infection, further work

across multiple hosts and pathogens combining multiple measures of both host and pathogen are needed.

One point of consideration to this pattern is that mortality patterns for the infected flies consisted of higher mortality close to after infection and lower mortality after this time point. To understand diet effects in these two time points, the effect of diet was analysed for the two time points separately. As many flies died in different diet groups in one of the time points, these results should be interpreted with caution, as they do not have the required sample size to understand these patterns with confidence. Infected individuals had very poor survival on low protein diets early on, however after this initial higher mortality, the effect of diet on survival was more linear compared to the control and injury group. This may be due to the very low sample size of flies that survived the initial mortality post-infection treatment affecting the survival estimates, for example the three individuals that survived infection post 10-days after infection on the lowest protein diet may not have ever been infected and therefore had similar lifespans to the uninfected groups. In addition, the large confidence intervals and the raw data suggest the spread of the available data was very variable. Overall, further experiments are needed to first determine whether individuals that survive the earlier higher mortality post-infection were infected, and second that there is a much higher sample size of individuals in this category to successfully analyse whether diet has a different effect on later-life survival of infected flies. Repeating this experiment with a less virulent pathogen may also aid in understanding these patterns further (see below).

Table 2.1: Effects of protein manipulation on survival with temperature or infection stressors in insect studies. For dietary manipulation, "Yeast restriction" is used when only yeast was restricted, so this dietary manipulation consisted of reduced calories and protein. For diets with higher survival with stressor, upwards arrows (↑) indicate survival was higher on higher protein diets, downwards signs (↓) indicate survival was higher on lower protein diets, and an equal sign (=) indicates that diet had no effect on survival post-infection.

Stressor	Species	Dietary protein manipulation	Survival measure	Diets with higher survival with stressor	References
Increasing temperature	Drosophila melanogaster	P:C	Lifespan	↓	Kim et al. 2020
Infection	Drosophila melanogaster	Addition of yeast on top of food	Number of days alive	↑	Le Rohellec and Le Bourg 2009
		Yeast restriction	24 hours	↑/=	Kutzer et al. 2018
		P:C or yeast restriction	Up to 160 hours	↓	Lee et al. 2017
		P:C	16 days	↓	Ponton et al. 2020
	27 species of Drosophilidae	P:C	20 days	=	Roberts and Longdon 2020

	Bactrocera tryoni Queensland fruit fly	P:C (liquid)	9 days	↓	Dinh et al. 2019
	Blatta orientalis cockroach	P:C	6 days	=	Sieksmeyer et al. 2019
	Nicrophorus vespilloides burying beetle	Protein:fat	22 days	↓	Miller and Cotter 2018
	Spodoptera littoralis caterpillars	P:C	Larval performance	↑	Lee et al. 2006
		P:C	Increased time to death	↑	Cotter et al. 2019; Wilson et al. 2020
	Spodoptera exempta caterpillars	P:C	Larval performance	↑	Povey et al. 2009, 2014

A further consideration in the survival patterns is the use of *P. entomophila* itself. We used a comparatively low dose of *P. entomophila*, as infection with *P. entomophila* can be very lethal at higher doses (reviewed in Dieppois et al., 2015). Due to logistic reasons, infection was completed systemically, however systemic infection can cause more variable bacterial loads compared to other methods (Troha & Buchon, 2019). Infecting flies in the thorax may have further affected mortality patterns due to this type of systemic infection increasing bacterial loads in comparison to the abdomen (Chambers et al., 2014). Finally, using an outbred population of *D. melanogaster* may add further variability. Further testing using additional pathogens which are not as lethal should be completed, as a higher dose could be used. Different pathogens can also introduce different patterns in bacterial persistence or clearance (see e.g. Duneau et al., 2017; Regoes et al., 2021). Using different pathogens would also allow investigating how the same diets affect survival post-infection mortality patterns and long-term survival. Altering the infection method and using a single strain of *D. melanogaster* would allow for better understanding about how diet influences survival post-infection and its generality.

Lifetime reproduction was maximised at intermediate protein levels, although at a slightly higher protein level than lifespan, a result which has been seen in other studies (Lee et al. 2008; Harrison et al. 2014; Jensen et al. 2015, but see Carey et al. 2008; Moatt et al. 2019). The decline in egg production with higher protein was not as steep in the early-life model, or in the model accounting for lifespan. Regardless of stress treatment, we saw the same patterns of highest egg counts on intermediate protein. Infection reduced egg production, as seen in many studies focusing on the reproduction-immunity trade off (reviewed in Schwenke et al., 2016). If lifetime reproduction models included lifespan, or only early-life reproduction was considered, there was no difference in reproduction between the stress treatments. This suggests that the pattern of lower lifetime reproduction in infected flies is most likely due to infected flies having shorter lifespans. Similar to our results, yeast restriction in *D. melanogaster* had a larger effect on early-life egg

production than infection (Kutzer & Armitage, 2016b; Kutzer et al., 2018). Contrary to our results, immune response activation can reduce reproduction when diet is limited (Stahlschmidt et al., 2013; Hudson et al., 2019), for example oral infection with *Pseudomonas aeruginosa* increased early-life egg production but only on higher protein diets (Hudson et al., 2019). Therefore, the methods of infection or the particular host-pathogen system may have an effect on the response of host reproduction on different diets.

The patterns of reproductive ageing involved complex interactions between diet and stress treatment. Broadly, there were similar ageing patterns across treatments and diets, with an increase in egg production followed by a peak and then diminishing egg numbers, as seen in other experiments (Carey et al. 2008; Le Rohellec and Le Bourg 2009). These peaks were higher for the high protein diets (but not necessarily the highest), most likely due to the requirement of protein for egg production (Wheeler, 1996; Mirth et al., 2019). Diets with low protein (e.g. 3 to 18% protein (1:26 to 1:4 P:C)) had the slowest rate of decline in egg production with age. This could simply be a result of individuals on high protein diets having much higher egg production earlier in life and thus a greater potential decline than on low protein diets. Individuals on high protein diets declined rapidly in egg production early in life before the rate of decline reduced to that of individuals on lower protein diets later in life, suggesting there is an initially higher rate of ageing on higher protein diets. Additionally, the control flies had a more linear decline in egg laying, suggesting that injury and infection might slightly delay egg production. Previous studies have also found ageing in female reproduction was quicker on higher protein with various diet manipulations (Carey et al. 2008; Le Rohellec and Le Bourg 2009; Jensen et al. 2015; Moatt et al. 2019, but see Maklakov et al. 2009). Overall, these similarities across studies suggest diet interacts with reproductive ageing in a broadly similar way across species.

Other than ageing in reproduction, we also investigated ageing in traits that are not implicated in the survival-reproduction trade-off, as delayed ageing is a known DR response (e.g. Ingram et al., 1987; Le Rohellec & Le Bourg, 2009;

Mattson et al., 2001; Regan et al., 2016; Rera et al., 2012). Ageing in negative geotaxis (NG) was delayed on lower protein diets, as has been found in another study limiting the addition of live yeast on food (Le Rohellec & Le Bourg, 2009). We did not see effects of stress treatment on NG, in contrast to a study where infection reduced the NG response in one of two tested *D. melanogaster* genetic backgrounds (Linderman et al., 2012), suggesting variation in the response depending on the genetic background of the host. Given the flies used in our study are genetically heterogeneous, the patterns we observe should be representative of the average genotype in this population.

We also measured the loss of gut integrity of flies with age using a smurf assay, which has been found to be more common in flies on unrestricted diets (Rera et al., 2012; Regan et al., 2016). Unexpectedly, we saw higher numbers of smurfs with lower protein in the control and infection treatments, whilst in the injured treatment we saw higher numbers at more intermediate protein levels. However, for the injured flies the peak smurfs still appeared at relatively low protein diets and may be due to the low sample sizes, especially for the infected flies on low protein diets. One explanation is that the lowest protein diets may represent malnourished conditions, leading to an increase in the number of smurfs. Nonetheless, we would still expect a reduction in smurf numbers at intermediate protein. In addition, for infected flies, the high mortality at high and low protein levels may result in flies dying before reaching the age where smurfs start appearing. Although only flies that survived to day 18 are included, flies may still have differential survival close to this date without surviving to a time where they would have developed a smurf phenotype. As oral infection with *P. entomophila* is known to damage the gut (Chakrabarti et al., 2012; Dieppois et al., 2015), further work is required to understand why some infected individuals did not develop into smurfs. The major problem with the interpretation of these results is the very low number of smurfs, meaning these patterns may not be robust. We analysed the smurf trait as a binary variable, however smurfs can be scored as a continuous trait as all individuals develop the trait (Martins et al., 2018). By measuring the phenotype with only clear

smurfs counted, we may have missed some more subtle patterns. More work is required to understand how the relationship between protein restriction and the appearance of smurfs varies with exposure to injury and infection, especially with a higher sample size of flies.

2.6 Conclusion:

The addition of injury and infection did not remove the lifespan benefit of protein restriction, the reduction in reproduction, or the delay in reproductive ageing. Our study therefore provides no evidence to support the nutrient recycling hypothesis of the lifespan response to dietary restriction. Even though there were minor differences between stress treatments in the relationship between protein content of the diet and survival, the major pattern of survival being maximised at intermediate protein levels was maintained across stress treatments. With infection, survival was particularly poor on the lowest protein diets, especially close to the infection treatment, whilst in the other treatment groups this difference was not as dramatic. The explanation for this pattern requires further investigation. Further work is required to understand how survival patterns change with diet with surviving infection and later-life survival. Our results and those of other studies suggest that the resource reallocation hypothesis remains the best-supported evolutionary explanation for the lifespan benefit of dietary restriction.

Chapter 3:

Larval diet affects adult reproduction but not survival regardless of injury and infection stress in *Drosophila melanogaster*

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3.1 Abstract:

Early-life conditions have profound effects on many life-history traits. In particular, early-life diet affects both juvenile development, and adult survival and reproduction. Early-life diet also has consequences for the ability of adults to withstand stressors such as starvation, temperature and desiccation. However, it is less well known how early-life diet influences the ability of adults to respond to infection. Here we test whether varying the larval diet of female *Drosophila melanogaster* (through altering protein to carbohydrate ratio, P:C) influences the long-term response to injury and infection with the bacterial pathogen *Pseudomonas entomophila*. Given previous work manipulating adult dietary P:C, we predicted that adults from larvae raised on higher P:C diets would be more likely to survive infection and have increased reproduction, but shorter lifespans and an increased rate of ageing. For larval development, we predicted that low P:C would lead to a longer development time and lower viability. We found that early-life and lifetime egg production were highest at intermediate to high larval P:C diets, but there was no effect of larval P:C on adult survival. Larval diet had no effect on survival or reproduction post-infection. Larval development was quickest on intermediate P:C and egg-to-pupae and egg-to-adult viability were higher on higher P:C. Overall, despite larval P:C affecting several traits measured in this study, we saw no evidence that larval P:C altered the consequence of infection or injury for adult survival and early-life and lifetime reproduction. Taken together, these data suggest that larval diets appear to have a limited impact on adult response to infection.

3.2 Introduction:

Early-life conditions are important in determining many key life-history traits (reviewed in Metcalfe and Monaghan, 2001, 2003). In particular, diet in early-life has been shown to have profound effects on later life-history traits such as survival and reproduction, and poor early nutrition can have costs associated with catch-up growth in adulthood (reviewed in Metcalfe and Monaghan, 2001, 2003). Nutrition is also important for the ability of an organism to respond to a number of key environmental stresses such as infection or temperature stress, as has been demonstrated in both juveniles (e.g. Lee *et al.*, 2006; Venesky *et al.*, 2012; Kutz, Sgrò and Mirth, 2019) and adults (e.g. Peck, Babcock and Alexander, 1992; Kim, Jang and Lee, 2020; Ponton *et al.*, 2020). However, work on the effect of early-life diet on adult responses to environmental stress is much more limited (but see e.g. Andersen et al. 2010; Kelly and Tawes 2013; Knutie et al. 2017). To investigate the long-term effects of early-life diet on adult traits and infection stress resistance, here we combine multiple larval diets, apply injury and infection to the adults and measure both larval and adult life-history responses in *Drosophila melanogaster.*

A vast literature exists using various approaches to manipulate diet and investigate the consequences of these manipulations (reviewed in Simpson and Raubenheimer, 2012). A particularly well-investigated manipulation is adult dietary restriction (DR), the restriction of calories or a particular nutrient without malnutrition, which has been shown to increase lifespan, delay ageing and reduce reproduction across a wide range of species (e.g. Mair and Dillin, 2008; Simpson *et al.*, 2017). Recent evidence suggests that this effect is mostly driven by changes in the protein to non-protein ratio of the diet, often protein to carbohydrate (P:C) ratios, particularly in insects (e.g. Lee *et al.*, 2008; Simpson *et al.*, 2017, but see Speakman, Mitchell and Mazidi, 2016). Regarding the effects of juvenile diet on juvenile and adult traits, there have been many studies testing the effects of caloric content (e.g. May, Doroszuk and Zwaan, 2015; Adler, Telford and Bonduriansky, 2016; House *et al.*, 2016; Littlefair and Knell, 2016; Hooper *et al.*, 2017; Krittika, Lenka and Yadav, 2019). As it has become clearer that macronutrient content is

more important than total caloric content, recent work has shifted to testing how the macronutrient composition of the juvenile diet may affect both juvenile and adult traits (reviewed in Nestel *et al.*, 2016). However, these studies often do not consider additional stressors (but see e.g. Andersen *et al.*, 2010; Kelly and Tawes, 2013; Pascacio-Villafán *et al.*, 2016).

Changing juvenile diet has been shown to alter the rate and success of the developmental period in both holometabolous (reviewed in Nestel *et al.*, 2016) and hemimetabolous insects (e.g. Hunt *et al.*, 2004; Kelly and Tawes, 2013; Houslay *et al.*, 2015). In general, juveniles on higher or intermediate P:C diets have a quicker development rate and improved development success (e.g. Matavelli *et al.*, 2015; Rodrigues *et al.*, 2015; Silva-Soares *et al.*, 2017, but see Cordes *et al.*, 2015; Houslay *et al.*, 2015; Davies *et al.*, 2018; Gray, Simpson and Polak, 2018; Kim *et al.*, 2019). In holometabolous insects, larvae have to pass several size assessment thresholds for successful pupation, and it has been suggested larvae feed until they have enough resources for metamorphosis and to survive the non-feeding state of pupation (reviewed in Mirth and Riddiford, 2007; Nestel *et al.*, 2016). As amino acids from protein in the diet signal a cell cycle for growth of tissues (Britton & Edgar, 1998; Colombani et al., 2003), and larvae do not develop on diets lacking in essential amino acids (e.g. Chang, 2004), it seems that higher larval P:C diets facilitate quicker growth and accumulation of essential resources that allow successful development into adulthood. There may be an upper limit after which increasing P:C has detrimental effects, potentially due to toxic effects of protein metabolism (Fanson et al., 2012), for example the accumulation of toxic wastes in food (reviewed in Simpson and Raubenheimer, 2009) or the highest P:C diets being limiting in carbohydrates, but the exact reasons are currently unknown.

Early-life diet has also been shown to have important consequences for many adult life-history traits, including reproduction, lifespan and ageing (reviewed in Metcalfe and Monaghan, 2001). In insects, measures of both early-life and lifetime egg production peak on higher or intermediate larval P:C diets (e.g. Rodrigues *et al.*, 2015; Silva-Soares *et al.*, 2017; Duxbury and Chapman, 2019, but

see Matavelli *et al.*, 2015). For lifespan, results of early-life dietary manipulation in insects are mixed, with lifespan being maximised at different P:C levels, and even no effect of P:C depending on the study (e.g. Runagall-McNaull, Bonduriansky and Crean, 2015; Stefana *et al.*, 2017; Davies *et al.*, 2018; Duxbury and Chapman, 2019). Indeed, a recent meta-analysis showed no consistent effect of early-life diet on adult lifespan across taxa (English & Uller, 2016). The age-related decline in various traits may also be altered by larval diet, however the direction of the effect is again unclear, with higher P:C or calorie diets leading to quicker, slower or having no effect on ageing (Tu & Tatar, 2003; May et al., 2015; Adler et al., 2016; Hooper et al., 2017). The effect of larval diet on adult reproduction may be a result of adults being able to use nutrient stores of, for example, protein or lipids in body tissues, including the fat body and haemolymph (reviewed in Boggs, 2009; Nestel *et al.*, 2016). However, it is less clear how these stored resources could affect lifespan. Potential explanations for inconsistencies in results across studies and life-history traits include that stored nutrients may trade-off between different adult life-history traits in an environment or species-specific manner, that juvenile diet effects may be dependent on adult food environment, or that storage of nutrients can be re-allocated in adulthood, for example by reabsorption of flight muscles (reviewed in Boggs, 2009; Nestel *et al.*, 2016). Overall, it seems that increasing P:C in the larval diet increases reproduction and juvenile diet often has effects on adult lifespan, but the directionality of the effects are inconsistent.

Despite the wealth of information on how larval diet affects multiple adult traits, studies focusing on adult stress resistance are rarer, despite the likelihood that stress resistance is a key trait in natural populations (e.g. Hoffman and Hercus, 2000; Van Voorhies, Fuchs and Thomas, 2005; Kawasaki *et al.*, 2008; Adamo, 2020). Some data exist on a small number of environmental stressors including temperature, desiccation and starvation (Andersen et al., 2010; Pascacio-Villafán et al., 2016; Davies et al., 2018), however the direction of effects are often mixed and potentially stress-specific. Particularly poorly studied is the effect of larval diet on adult infection response. In *Anopheles gambiae,* melanising ability decreased with

severity of larval calorie restriction (Suwanchaichinda & Paskewitz, 1998). Female *Gryllus texensis* crickets on lower P:C as nymphs and adults survive better over five days post-infection (Kelly & Tawes, 2013). As this study changed both adult and larval diet, it is not possible to disentangle the effect of larval diet alone. Without a direct immune stress, there is evidence for differential adult response to immune challenge due to larval diet. For example, the production of antimicrobial peptides (AMPs) in adults decreased with lower larval P:C in *D. melanogaster* (Fellous & Lazzaro, 2010). For other immune response measures, in *Lestes viridis* damselflies, lower calories and starvation led to reduced phenoloxidase (PO) activity and haemocyte numbers/levels in adults (Rolff et al., 2004; De Block & Stoks, 2008). However, to our knowledge, no study to date has tested the effect of larval dietary P:C on adult life-history traits when exposed to infection or injury stress.

Several hypotheses have been put forward to explain the effect of larval diet on adult survival post-infection. These include increased stress response capability due to overall better body condition, and increased investment into immunity, either through the growth of specific tissues, or through increased availability of limiting nutrients (Fellous & Lazzaro, 2010). The first suggestion is supported by studies where both immune response and body condition are lower with starvation (Suwanchaichinda & Paskewitz, 1998; Rolff et al., 2004), but the independent effects are difficult to separate (Fellous & Lazzaro, 2010). The second hypothesis is supported by studies where indicators of immune response increase in adults or pupae outside of effects on general body condition with a diet higher in P:C in *D. melanogaster* (Fellous & Lazzaro, 2010) or plant diets of worse quality in *Epirrita autumnata* moths (Klemola et al., 2007). In general protein seems to be an important nutrient in relation to survival post-infection (e.g. Lee et al. 2006; Povey et al. 2009, 2014; Cotter et al. 2011, 2019; Chapter 2), suggesting individuals developing on higher larval P:C diets should have improved resistance to infection.

To test the effects of larval P:C on larval and adult life-history and adult survival post-infection, we reared larvae on various P:C diets and exposed female adults to injury and infection stress (with a bacterial pathogen,

Pseudomonas entomophila). For the larvae, we measured development time to adulthood and measures of viability (egg-to-pupae, egg-to-adult and pupae-to-adult viability). For the adults, we measured the key life-history traits of survival and reproduction. We predicted that low P:C larval food would lead to longer development time and lower viability across all stages. If larval diet affects adult life-history traits independent of adult food, we predicted a similar effect to that observed when P:C ratio is manipulated in adults (e.g. Lee et al. 2008; Jensen et al. 2015; Chapter 2), with low larval P:C extending lifespan, and reducing reproduction and the senescent decline in egg laying. Conversely, if larval diet has no long-term effects on life-history traits, we would expect to see similar survival and reproduction patterns across all diets. As low P:C diets have been found to be especially detrimental for survival post-infection in this host-pathogen system (Chapter 2), we predicted that low larval P:C would reduce survival and reproduction to a greater extent in injured and infected flies than in control flies.

3.3 Methods:

3.3.1 Larval diets:

Larval diets consisted of a five P:C diets: 1:16 P:C (5% protein), 1:6 (14%), 1:2 P:C (31%), 1:1 (46%) and 2:1 P:C (61%) (see Chapter 1, section 1.11.2). These are a subset from ten diets used in Chapter 2.

3.3.2 Larval experimental methods:

D. melanogaster experimental individuals were from an outcross DGRP population (see Chapter 1, section 1.11.1 and Appendix A). From the 35th generation, we pipetted 5 µl of egg solution into each larval diet vial (following Clancy and Kennington, 2001) to establish density controlled groups of eggs (on average 50 (± 19) eggs). The mean volume of food in vials was 7.66 (± 0.58) ml. 30 vials of each diet were prepared in this way, and an additional 21 of the lowest P:C diet to ensure enough adults for adult collection. Due to very low egg counts in a small number of vials, a further 5-10 µl was added to these vials (17/171 vials, approximately 10%). For each vial, eggs were counted twice under a microscope to get an average egg count. Starting from experimental day 1 (one day after adding eggs to diets), vials were inspected daily for adult eclosion and the total number of adults eclosed per vial was recorded. The number of pupae per vial was counted once all adults had eclosed, based on pupal cases and undeveloped pupae.

3.3.3 Adult collection:

Eclosion began on experimental day 8, from which point onwards adults were counted and removed from vials twice a day, in the morning and evening. Pilot data suggested different development times on the different diets, and therefore for each diet adults were collected primarily across three days per diet, starting one day after adult eclosion began (Figure S3.1). In this way, adult females were collected across a total of six days across all diet treatments to create six blocks. Some diets with quicker development times required some adults to be collected on the fourth day to achieve sufficient sample sizes (Figure S3.1 and

Appendix C, Table S3.1; sample sizes = 18 to 40 adult flies per larval diet and stress treatment).

After adult collection, all flies were placed singly in vials containing standard Lewis medium in our laboratory, corresponding to the 1:6 P:C diet (14% protein diet, see Table 1.1, Chapter 1, 1.11.2), and were maintained on this diet for their remaining lifespan. Trays were rotated in the incubators daily to minimise microclimate effects. The day following adult collection, each female was provided with an age-matched male from the same outcross DGPR population. The female was left with the male for 24 hours to allow mating, following which the male was removed.

3.3.4 Stress treatments:

On the seventh day post-eclosion for each block, female flies were exposed to one of three stress treatments, as described in Chapter 1, section 1.11.3. Treatments for each block were done at the same time each day (around 14:00) to minimise time-of-day effects on immunity (e.g. Lee and Edery, 2008). After stress treatments, the fly was placed into a new vial containing modified Lewis medium (Lewis, 1960, see P:C 1:6 diet in Table 1.1, Chapter 1, section 1.11.2). The overnight *P. entomophila* bacterial solution was re-suspended in 30 ml Luria-Bertani (LB) medium in the morning and left to grow for three hours prior to dilution from a known OD value to correspond to an OD value of 0.001. This level is slightly lower than a previous experiment in adults (Chapter 2), as the diluted OD of 0.005 had much lower survival compared to the previous experiment (Appendix C, Figure S3.2). Each block's bacterial culture was established from a set of isogenic bacterial cultures grown overnight in LB medium, aliquoted in 20-25% glycerol 200 µl quantities and stored at -80°C. A subset of flies from each block of infections were plated on *Pseudomonas* isolating agar to confirm the infection treatments were successful (following Gupta *et al.*, 2017, see Appendix C).

3.3.5 Adult trait measurements:

The number of eggs a female laid was counted from day 2 onwards for each block. For the first 14 days, eggs were counted daily and females were tipped into new vials. Subsequently, egg counts were performed every second day and stopped on day 98 for logistical reasons. This is an accurate proxy for lifetime egg production, as females in a previous experiment with adult P:C diet manipulation laid on average 99.37% (± 2.34%) of their lifetime eggs by day 98 (Chapter 2). If a fly died on a day when eggs were not counted, an extra egg count was performed on the day of death. Survival was checked daily.

3.3.6 Statistical methods:

The data were analysed using R software, version 3.5.2, with analysis of survival patterns before and after 13 days analysed using version 4.0.5 (R Core Team, 2014). All graphs were drawn using ggplot2 (Wickham, 2016). All traits were analysed using generalised linear mixed models (GLMM). Models using a Poisson distribution were checked for zero inflation and overdispersion using the *DHARMa* package (Hartig, 2021). In all models, even though we altered the P:C ratio of diets, diet was analysed as a continuous covariate as the percentage of protein in the diet (Table 1.1, Chapter 1, section 1.11.2). To allow for non-linear effects, the quadratic term of protein percentage was also included. To avoid scaling errors, all continuous covariates were standardised to a mean of zero and a standard deviation of one. This was done separately for each test due to different sample sizes for different measures. Stress treatment was analysed as a categorical fixed effect. For all models with two-, or three-way interactions, for displaying summaries of LRT results of main effects or two-way interactions, parameter estimates and associated standard deviations are from separate models not including the associated two-, or three-way interactions. All model prediction plots were made with all random effects set to 1 and diets are shown as the percentage of protein in the diet. Full models without model simplification were used (see Chapter 2 statistical methods for discussion).

We analysed survival to a number of developmental stages: egg-to-pupa, pupa-to-adult and egg-to-adult using linear models assuming a Gaussian distribution. For egg-to-pupa and egg-to-adult survival, we included the number of eggs in each vial as a covariate to control for differences in initial egg number on how many pupae or adults developed in each vial. This is essentially the same as modelling viability as percentages or as a proportion, which is often done in other larval diet studies (e.g. Andersen *et al.*, 2010; Sentinella, Crean and Bonduriansky, 2013; Kutz, Sgrò and Mirth, 2019), however our method does not bound the data at 100% or 1. Similarly, for egg-to-pupa survival, we included the number of pupae in each vial as a covariate. All models were visually analysed for normality. Development time was analysed through GLMM with a Poisson error distribution using the lme4 R package (Bates et al., 2015) with the "bobyqa" optimiser with 100'000 iterations. We included vial as a random effect to account for any within vial effects, for example different larval densities and repeated measurements. For clarity of presentation, model predictions were made at either the average number of eggs or pupae per vial.

For adult survival, Kaplan-Meier survival curves were made using the survminer R package (Kassambara & Kosinski, 2018) with diet as a factor. As the survival data for the entire experiment did not conform to the assumptions of proportional hazards (global term of cox.zph function Chisq = 54.98, p = <0.001), we used an event history model following Moatt *et al.* (2019, Chapter 2), implemented through a binomial GLMM in the lme4 R package (Bates et al., 2015) with the "bobyqa" optimiser with 100'000 iterations. This model is similar to a Cox proportional hazards model, however the results in this case estimate a per day mortality risk, which we will refer to as mortality. Individuals in the dataset were scored daily as 0 for alive and once as 1 for dead. The model included day as a random effect to account for differences in survival between days, and individual ID to account for multiple measures of an individual. To confirm the results of the survival model, we further analysed the data as lifespan using a LMM with block as a random effect to account for differences between infection days. Even though

the data do not conform to the Cox proportional hazards assumptions (global term cox.zph = 55.22, p = <0.001), we checked for consistency with the result of the above analysis using a Cox proportional hazards model in the R Survival package (Therneau, 2015).

As the infected flies either died close to the infection treatment, or survived beyond this point, the survival data were analysed with two separate subsets. Day 13 post-eclosion was chosen as a cut-off point, as this was the first day that the number of dead flies was below two per day (see Appendix C, Figure S3.8 for the number of dead flies per day post-infection treatment). It should be noted that the number of flies per larval diet and stress treatment groups is often very low and therefore the results of these analyses should be interpreted with caution (see Appendix C, Table S3.4 for sample sizes per group). To analyse whether mortality patterns and relationships changed for infected flies dying in each group, an event history binomial model similar to the full dataset was used, where each fly was labelled as whether they died prior to or after 13 days post-eclosion. This category and its interactions with larval protein and its squared term were added in the model. For each subset separately, an event history binomial model, a linear model and a Cox proportional hazards models (although the data did not fit the assumption of proportional hazards, before day 13 cox.zph = 16.86, p = 0.03, after day 13 cox.zph = 19.15, p = 0.01) were used similar to the full subset. For the analysis of flies that died prior to day 13 post-eclosion, all flies that survived to day 13 were included in the analysis as censored data points.

For reproduction, various measures were analysed. As adult diet might have an increasing influence on the number of eggs produced as flies get older, for example due to compensatory feeding, we analysed measures of early-life egg production. We ran separate analyses on the number of eggs produced prior to stress treatments, seven days in total, and eggs produced in the seven days post stress treatments to test for early-life differences in reproduction and if these were affected by stress treatment. Only flies left alive on the last day of egg counts were included in these analyses. These data were analysed using a GLMM with a

negative binomial distribution and including a zero-inflation term with the glmmTMB R package (Brooks et al., 2017). Lifetime egg production (to day 98) was analysed with an identical model to the other reproduction models with all flies included, with an additional model including mean centered lifespan as a predictor. In this model, mean centered lifespan was included to account for selective disappearance and block was included as a random effect. To analyse daily egg production, all egg counts corresponding for a span of two days were divided by two and rounded down to the nearest integer to match earlier daily egg counts. Daily egg production was analysed using a GLMM with negative binomial distribution and including a zero-inflation term. As well as the fixed effects described above, age was included as a linear and non-linear term as well as the interactions between these and all other fixed effects. Individual ID and block were included as random effects.

3.4 Results:

3.4.1 Effects of larval nutrition on larval traits:

P:C of the larval diet had a significant effect on how many individuals developed from eggs to adults, where larvae reared on higher P:C were more likely to develop to adults (Figure 3.1A & Appendix C, Figure S3.3A; Table 3.1A; Protein = 2.20 (± 0.55), F = 15.90, p = <0.001). With higher numbers of eggs in a vial, more adults developed (Table 3.1A; Average number of eggs = 0.72 (± 0.03), F = 762.19, p = <0.001). Separating this result into effects on larval and pupal viability, there was a significant effect of P:C on the numbers of eggs developing into pupae (Figure 3.1B & Appendix C, Figure S3.3B; Table 3.1B; Protein = 1.79 (± 0.52), F = 9.13, p = 0.003; Average number of eggs = 0.86 (± 0.03), F = 1165.5, p = <0.001). There was a marginally non-significant effect of P:C on the number of adults developing from pupae (Figure 3.1C & Appendix C, Figure S3.3C; Table 3.1C; Protein = 0.70 (± 0.45), F = 3.81, p = 0.052). As expected, with more pupae in a vial, more adults developed (Table 3.1C; Pupae = 0.82 (± 0.02), F = 1228.5, p = <0.001).

P:C in the larval diet also had an effect on the development time to adulthood, with higher larval P:C resulting in shorter development time (Figure 3.1D & Appendix C, Figure S3.4, Table S3.2; Protein = -0.22 (± 0.01), Chi-squared = 191.75, p = <0.001). This relationship is quadratic, suggesting that intermediate P:C diets had a quicker development time in comparison to the high or low P:C diets, or that the rate of reduction in development time plateaued at the highest P:C diets (Figure 3.1D & Appendix C, Figure S3.4, Table 3.2; Protein^2 = 0.16 (± 0.01), Chi-squared = 183.61, p = <0.001). Vials with higher average number of eggs had a slightly longer development time (Table 3.2; Average number of eggs = 0.03 (± 0.01), Chi-squared = 26.80, p = <0.001).

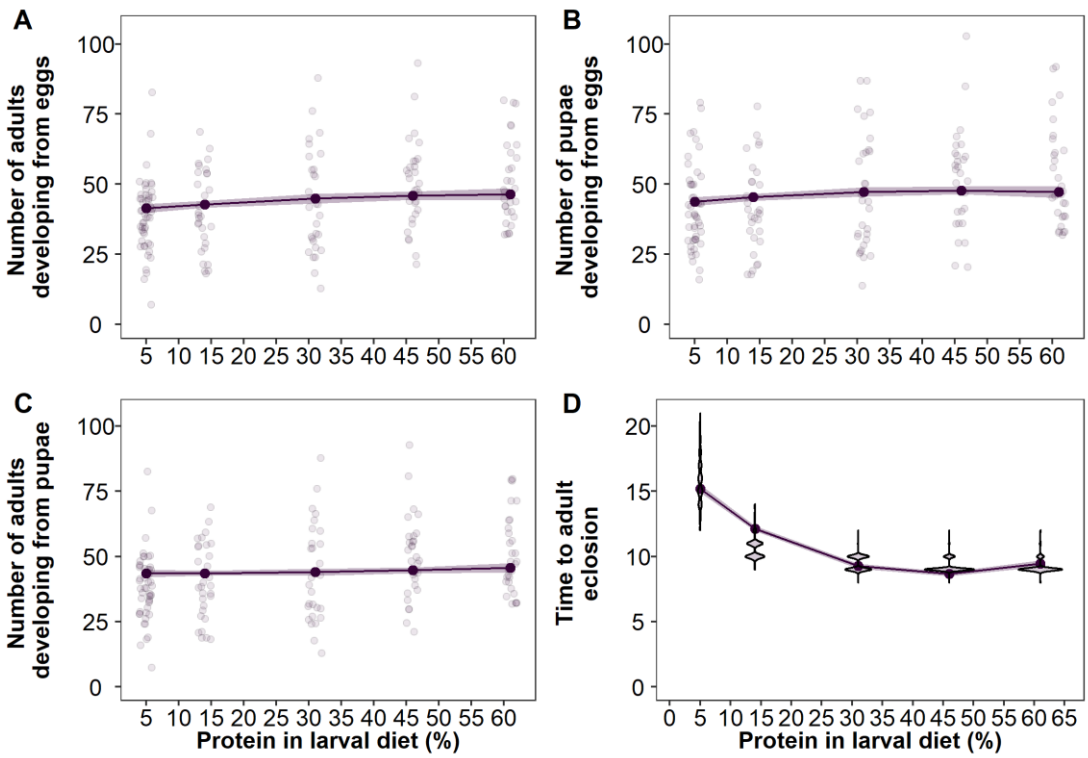

Figure 3.1: Model predictions of the effects of larval P:C (shown as the corresponding percentage of protein) on various larval traits: (A) the number of adults developing having controlled for the number of eggs laid (50 (± 19) eggs on average); (B) the number of pupae eclosing having controlled for the number of eggs laid (50 (± 19) eggs on average); (C) the number of adults developing having controlled for the number of pupae formed (46 (± 18) pupae on average); and (D) the average time taken for adult eclosion. All predictions are based on either vials starting with the overall mean number of eggs (50 (± 19) eggs) (A, B, D), or the overall mean number of pupae (46 (± 18) pupae) (C). Shaded areas are 95% confidence intervals. Protein and protein^2 are mean centered to standard deviation of 1. See Figure S3.3 for viability data as percentages. Additional data points (A, B, C) or violin plot (D) show raw data values, however note that these data points do not include standardisation to the original number of eggs or pupae, which is included in the model predictions.

Table 3.1: Model summary of a Gaussian linear model of the effects of protein in larval diet and the number of eggs laid in the vial (averaged over two counts, see methods) on the number of adults developing per vial (A); the number of eggs laid in the vial on the number of pupae developing per vial (B); and the number of pupae in the vial on the number of adults developing per vial (C). Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded.

(A) Number of adults developing from eggs:					
	Estimate	Standard error	Df	F	Pr (>F)
Intercept	8.27	1.54			
Average number of eggs	**0.72**	**0.03**	**1**	**762.19**	**<0.001**
Protein	**2.20**	**0.55**	**1**	**15.90**	**<0.001**
Protein^2	-0.66	0.69	1	0.91	0.34
(B) Number of pupae developing from eggs:					
	Estimate	Standard error	Df	F	Pr (>F)
Intercept	3.99	1.47			
Average number of eggs	**0.86**	**0.03**	**1**	**1165.5**	**<0.001**
Protein	**1.79**	**0.52**	**1**	**9.13**	**0.003**
Protein^2	-1.05	0.66	1	2.57	0.11
(C) Number of adults developing from pupae:					
	Estimate	Standard error	Df	F	Pr (>F)
Intercept	6.15	1.27			
Pupae	**0.82**	**0.02**	**1**	**1228.5**	**<0.001**
Protein	0.70	0.45	1	3.81	0.052
Protein^2	0.31	0.56	1	0.31	0.58

Table 3.2: Model summary of a Poisson model of the effects of protein in larval diet and the average number of eggs laid in the vial on the number of days until adult eclosion. Vial ID was fitted as a random effect. Protein, protein^2 and average egg counts are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded.

	Estimate	Standard error	Z value	Df	Chisq	Pr (>Chisq)
Intercept	2.23	0.01	206.87			
Protein	**-0.22**	**0.01**	**-35.32**	**1**	**191.75**	**<0.001**
Protein^2	**0.16**	**0.01**	**18.33**	**1**	**183.61**	**<0.001**
Average number of eggs	**0.03**	**0.01**	**5.39**	**1**	**26.80**	**<0.001**

3.4.2 Effects of larval nutrition on adult traits and survival after stress:

P:C of larval diet had no effect on adult mortality regardless of stress treatment (Figure 3.2 &3.3A; Table 3.3). Stress treatment had a significant effect on mortality, where infected flies had a higher risk of death (Figure 3.2 & 3.3A; Table 3.3A; Treatment Chi-squared = 76.67, p = <0.001; Infection = 1.18 (± 0.13); Injury = 0.24 (± 0.17)). Analysing the survival data as lifespan, the same patterns were observed, where stress treatment had a significant effect on lifespan, with infected flies having shorter lifespans (Appendix C, Figure S3.5 & S3.6; Table S3.2; Treatment Chi-squared = 99.28, p = <0.001; Infection = -1.00 (± 0.10); Injury = -0.10 (± 0.10)). The results of a Cox proportional hazards model show the same patterns (Appendix C, Figure S3.7; Table S3.3).

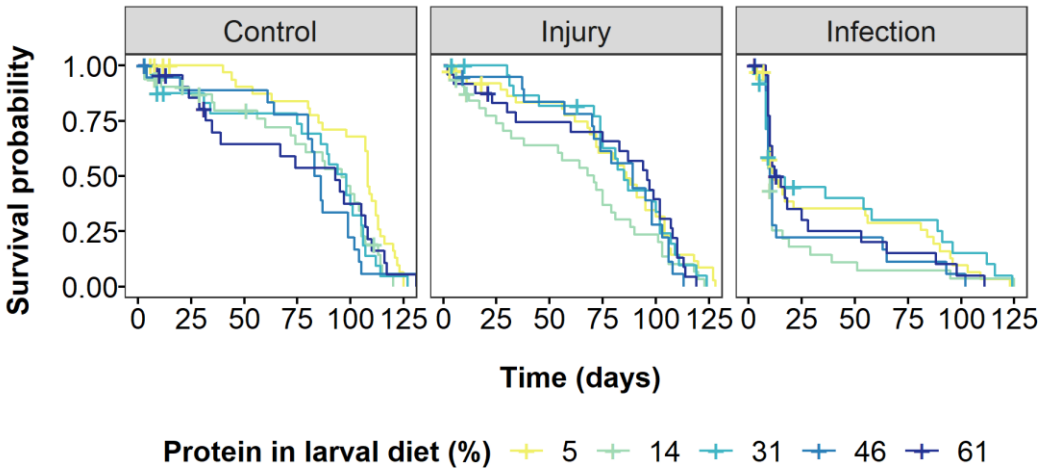

Figure 3.2: Effects of protein in larval diet on survival of adult flies infected with a bacterial pathogen ("Infection"), injured by a pinprick ("Injury") or with no treatment ("Control"). Survival is shown as Kaplan-Meier curves for each stress and diet treatment groups. Plus signs (+) indicate censored data points.

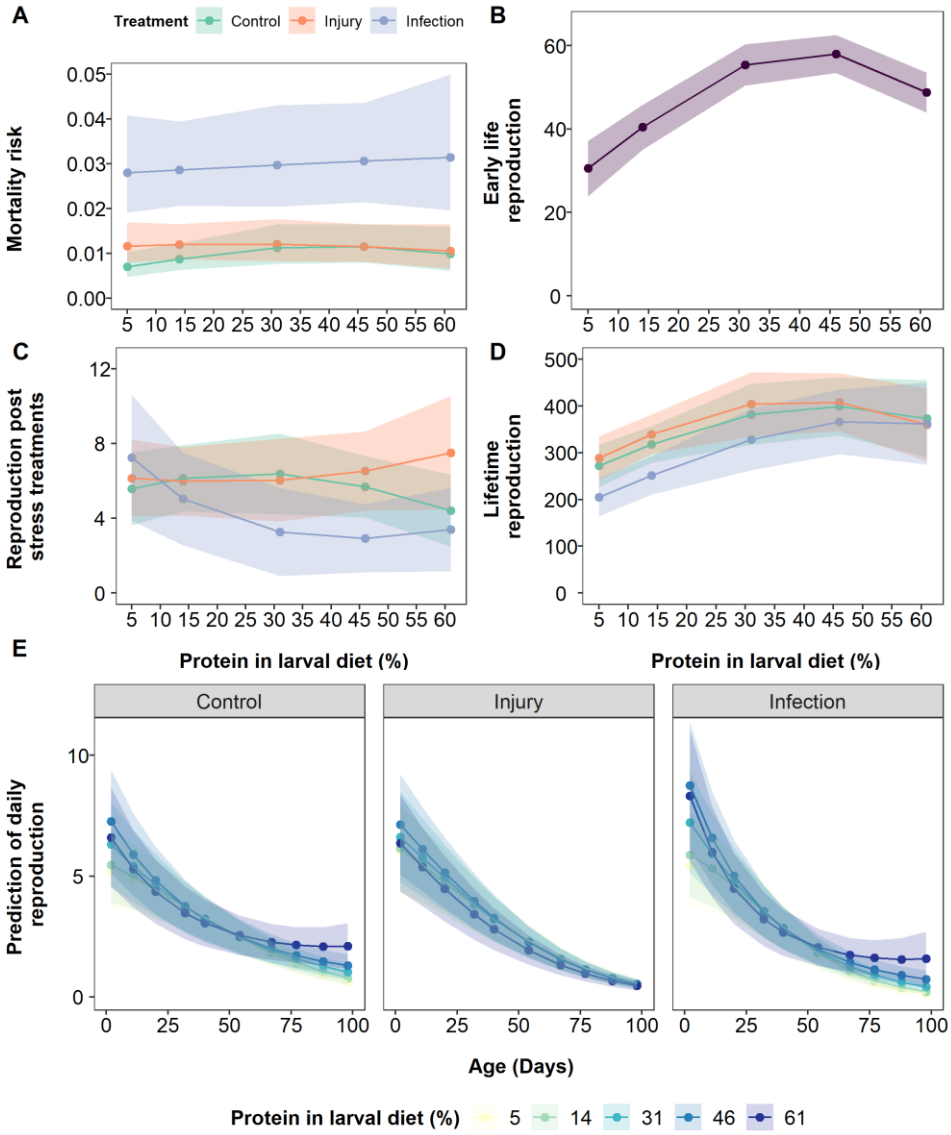

Figure 3.3: Model predictions of the effects of larval diet P:C (shown as the corresponding percentage of protein) and adult stress treatment on various adult life-history traits: (A) per day mortality risk; (B) egg production in the first 7 days of adulthood (prior to stress treatments); (C) egg production across the 7 days after stress treatments; (D) lifetime egg production (up to day 98); (E) reproductive ageing in terms of daily egg production. Adult flies were infected with a bacterial pathogen (blue data points and lines in A, C, D), injured by a pinprick (orange data points and lines in A, C, D) or with no treatment (green data points and lines in A, C, D). Lifespan is accounted in the model to account for selective disappearance in (D). Shaded areas are 95% confidence intervals. Protein and protein^2 (A-E), and age and age^2 (E) are mean centered to standard deviation of 1.

Table 3.3: Summary of a binomial event history model analysing effects of P:C in larval diet and stress treatments on mortality risk per day post infection treatment with (A) main effects parameter estimates and associated LRT tests and (B) for the full model. Protein and protein^2 are mean centered to standard deviation of 1. The models include day and individual ID as random effects. Significant results below significance level $\alpha = 0.05$ are bolded. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	Z value	Df	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:						
Injury treatment	**0.24**	**0.13**	**-1.87**	**2**	**76.67**	**<0.001**
Infection treatment	**1.18**	**0.13**	**9.04**			
Protein	0.05	0.05	0.98	1	0.88	0.35
Protein^2	-0.07	0.07	-1.01	1	0.98	0.32
(B) Full model parameter estimates and LRT test values for interactions:						
Intercept	-4.52	0.19	-23.34			
Injury treatment	0.12	0.21	0.57			
Infection treatment	1.02	0.21	4.94			
Protein	0.23	0.11	2.13			
Protein^2	-0.17	0.12	-1.40			
Injury:Protein	-0.24	0.15	-1.60	2	2.52	0.28
Infection:Protein	-0.18	0.15	-1.22			
Injury:Protein^2	0.12	0.17	0.74	2	0.97	0.62
Infection:Protein^2	0.17	0.17	1.00			

As infected flies had high mortality close to the infection treatment, the survival data for all flies was divided into flies that either survived this initial period or died close to infection. A cut-off point of 13 days post-eclosion was chosen, as at 13 days post-eclosion the number of flies dead per day was below 3 flies for the first time (13 days post-eclosion is 6 days post-stress treatments, see Appendix C, Figure S3.8 for number of dead infected flies per day). It should be noted that the following analyses should be interpreted with caution, as some of the diet by stress treatment combinations only consist of a few flies, many as low as 2, which influences the inferred patterns (see Appendix C, Table S3.4 for sample sizes). This experiment should be repeated with a higher sample size to test these patterns in a statistically robust way. From an event history binomial model where infected flies were labelled as dying before or after 13 days post-eclosion, infected flies had higher levels of mortality prior to 13 days post-eclosion, however larval diet had no effect on mortality in either time-point (Figure 3.4, Table 3.4; Dying before or after 13 days post-eclosion Chi-squared = 126.82, p = <0.001; Dying post-13 days = -3.87 ($\pm$ 0.52)).

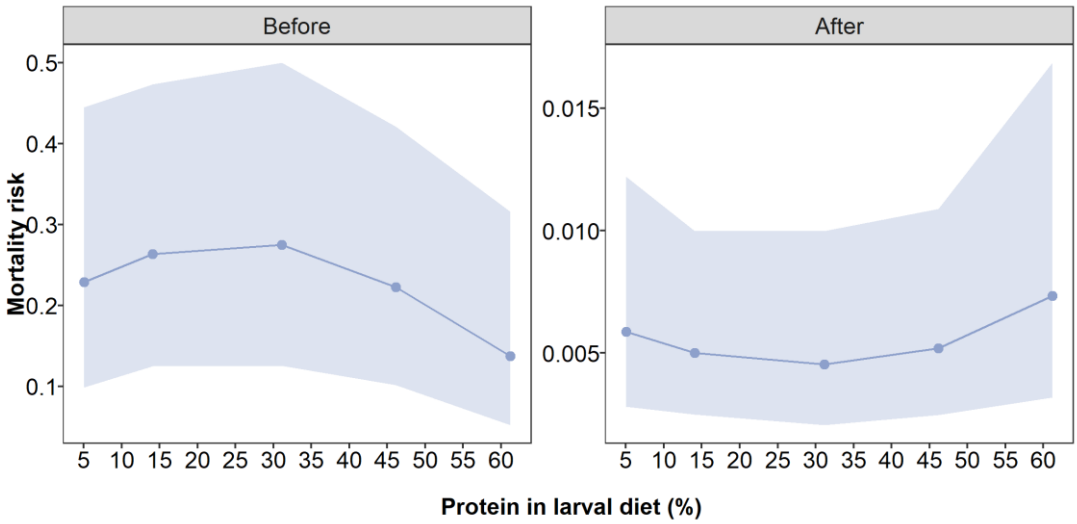

Figure 3.4: Model predictions from an event history binomial model for the effect of P:C in larval diet on mortality risk per day of flies infected with a bacterial pathogen, for flies which died prior to ("Before") and after 13 days post-eclosion treatment ("After"). In the binomial model, for each day each fly was coded as 0 for alive and 1 for dead. Protein and protein^2 are mean centered to standard deviation of 1. Shaded areas are 95% credible intervals. The two time points have different y-axis scales for ease of interpretation.

Table 3.4: Summary of a binomial model analysing effects of P:C in larval diet and stress treatments on mortality risk of adult flies that died either prior to or after 13 days post-infection treatment with (A) main effects parameter estimates and associated LRT tests and (B) for the full model. Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level α = 0.05 are bolded. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	Z value	Df	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:						
Protein	-0.08	0.10	-0.76	1	0.58	0.45
Protein^2	-0.03	0.14	-0.18	1	0.03	0.86
Dying post-13 days	**-3.87**	**0.52**	**-7.48**	**1**	**126.82**	**<0.001**
(B) Full model parameter estimates and LRT test values for interactions:						
Intercept	-0.95	0.49	-1.93			
Protein	-0.05	0.18	-0.30			
Protein^2	-0.29	0.21	-1.39			
Dying post-13 days	-4.44	0.62	-7.18			
Protein:Dying post-13 days	0.02	0.25	0.06	1	0.004	0.95
Protein^2:Dying post-13 days	0.49	0.28	1.72	1	2.99	0.08

When analysing survival patterns for each subset separately, for flies that died prior to 13 days post-stress treatment, infected flies had higher mortality, however larval diet had no significant effect on adult mortality (Figure 3.5, Table 3.5; Treatment Chi-squared = 124.47, p = <0.001; Infection = 2.70 (± 0.37)). Analysing the survival data with a Cox proportional hazards model, although the assumption of proportional hazards was not met, similar results were found (Appendix C, Figure S3.9, Table S3.5). Analysing the data as lifespan, stress treatment had a significant effect on lifespan, however infected flies had a higher lifespan compared to the control and injured flies (Appendix C, Figure S3.10, Table S3.6). This is however most likely due to the relatively few flies that died in the control and injury groups having a high variation in lifespan data, whereas there were many more infected flies with lifespans below 13 days post-eclosion and estimates could be more accurate (see wide standard deviations in Appendix C, Figure S3.10, and sample sizes in Table S3.4). As the models analysing survival data account for flies that were still alive at the end of day 13 post-eclosion, most likely similar patterns were not found to the lifespan analysis where only the number of deaths could be analysed.

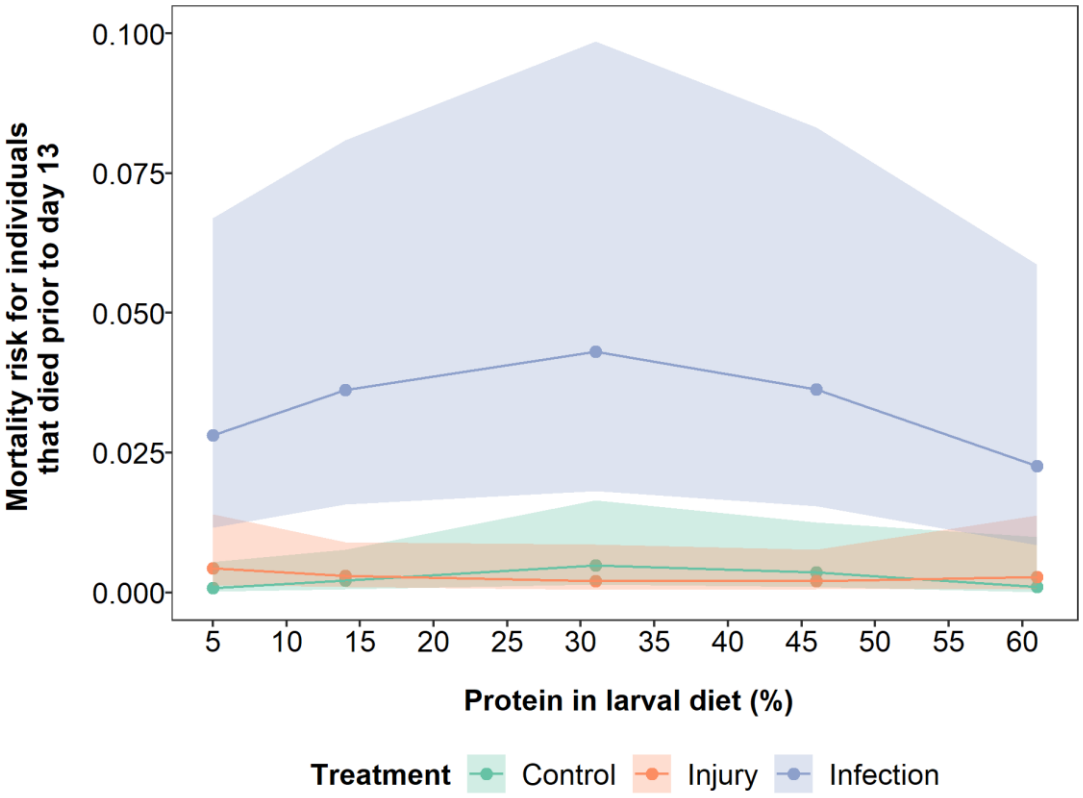

Figure 3.5: Model predictions from an event history binomial model for the effect of P:C in larval diet on mortality risk per day of flies that died prior to 13 days post-eclosion for flies infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines). In the binomial model, for each day each fly was coded as 0 for alive and 1 for dead. Protein and protein2 are mean centered to standard deviation of 1. Shaded areas are 95% credible intervals.

Table 3.5: Summary of a binomial event history model analysing effects of P:C in larval diet and stress treatments on mortality risk per day post infection treatment for flies that died before 13 days post-stress treatment with (A) main effects parameter estimates and associated LRT tests and (B) for the full model. Protein and protein^2 are mean centered to standard deviation of 1. The models include day and individual ID as random effects. Significant results below significance level α = 0.05 are bolded. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	Z value	Df	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:						
Injury treatment	**0.29**	**0.46**	**0.62**	**2**	**124.47**	**<0.001**
Infection treatment	**2.70**	**0.37**	**7.30**			
Protein	-0.05	0.10	-0.51	1	0.25	0.62
Protein^2	-0.28	0.15	-1.94	1	3.64	0.06
(B) Full model parameter estimates and LRT test values for interactions:						
Intercept	-5.40	0.51	-8.66			
Injury treatment	-0.74	0.75	-0.99			
Infection treatment	2.29	0.50	4.54			
Protein	0.56	0.50	1.12			
Protein^2	-0.92	0.55	-1.69			
Injury:Protein	-0.89	0.61	-0.46	2	2.28	0.32
Infection:Protein	-0.48	0.52	-0.93			
Injury:Protein^2	1.21	0.70	1.74	2	3.21	0.20
Infection:Protein^2	0.62	0.57	1.09			

For flies that survived at least 13 days post-eclosion, infected flies had higher mortality, and larval diet had no effect on adult mortality (Figure 3.6, Table 3.6; Treatment Chi-squared = 9.60, p = 0.008; Infection = 0.55 (± 0.17)). Analysing the data with a Cox proportional hazards model, again where the assumptions of proportional hazards were not met, there was a significant interaction between infection and the quadratic effect of protein in larval diet, suggesting that infected flies had lowest adult mortality at intermediate larval protein diets (Appendix C, Figure S3. 11, Table S3.7; Infection:Protein^2 HR = 1.64 (95% CI = 1.03 to 2.59). Analysing the data as lifespan, infection reduced survival in flies that survived at least to 13 days post-eclosion (Appendix C, Figure S3.12, Table S3.8). Although the models differed slightly, they all show qualitatively similar results where infection reduces survival. A larger sample size is required to distinguish whether protein in larval diet affected lifespan differently with infected flies, or whether this is an effect of the model to the low sample size.

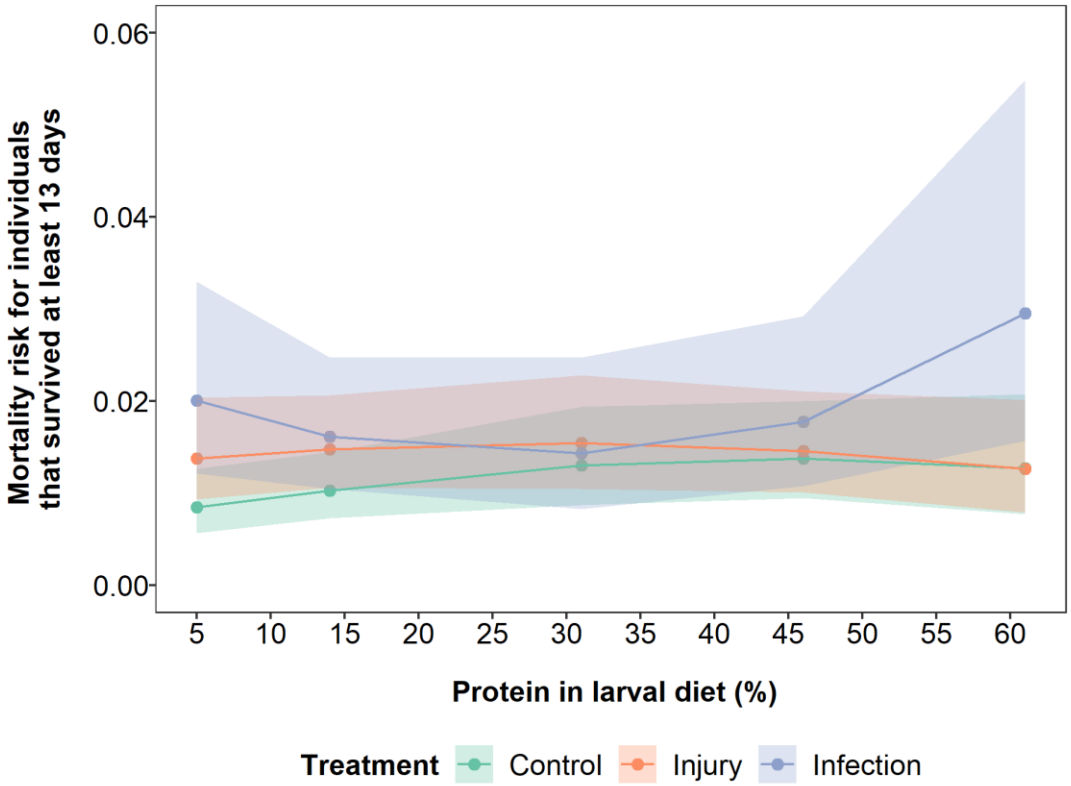

Figure 3.6: Model predictions from an event history binomial model for the effect of protein restriction on mortality risk per day of flies that survived at least 13 days post-eclosion of flies infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines). In the binomial model, for each day each fly was coded as 0 for alive and 1 for dead. Protein and protein2 are mean centered to standard deviation of 1. Shaded areas are 95% credible intervals.

Table 3.6: Summary of a binomial event history model analysing effects of P:C in larval diet and stress treatments on mortality risk per day post infection treatment for flies that died before 13 days post-stress treatment with (A) main effects parameter estimates and associated LRT tests and (B) for the full model. Protein and protein^2 are mean centered to standard deviation of 1. The models include day and individual ID as random effects. Significant results below significance level $\alpha = 0.05$ are bolded. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	Z value	Df	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:						
Injury treatment	**0.25**	**0.13**	**1.94**	**2**	**9.60**	**0.008**
Infection treatment	**0.55**	**0.17**	**3.24**			
Protein	0.07	0.06	1.25	1	1.40	0.24
Protein^2	-0.03	0.08	-0.40	1	0.14	0.70
(B) Full model parameter estimates and LRT test values for interactions:						
Intercept	-4.37	0.20	-21.60			
Injury treatment	0.22	0.22	1.00			
Infection treatment	0.13	0.29	0.46			
Protein	0.23	0.11	2.11			
Protein^2	-0.13	0.13	-1.07			
Injury:Protein	-0.22	0.15	-1.34	2	2.36	0.31
Infection:Protein	-0.26	0.20	-1.33			
Injury:Protein^2	0.05	0.17	0.28	2	3.63	0.16
Infection:Protein^2	0.42	0.22	1.91			

Larval P:C had a significant effect on early-life egg production prior to stress treatments, where increasing larval P:C increased early-life egg production which then levelled off at very high P:C diets (Figure 3.3B & 3.7; Table 3.7; Protein = 0.29 (± 0.06), chi-squared = 3.71, p = 0.054; Protein^2 = -0.20 (± 0.04), p = <0.001). There were no significant effects of larval diet or stress treatments on egg production in the seven days following stress treatments (Figure 3.3C & 3.8; Table 3.8).

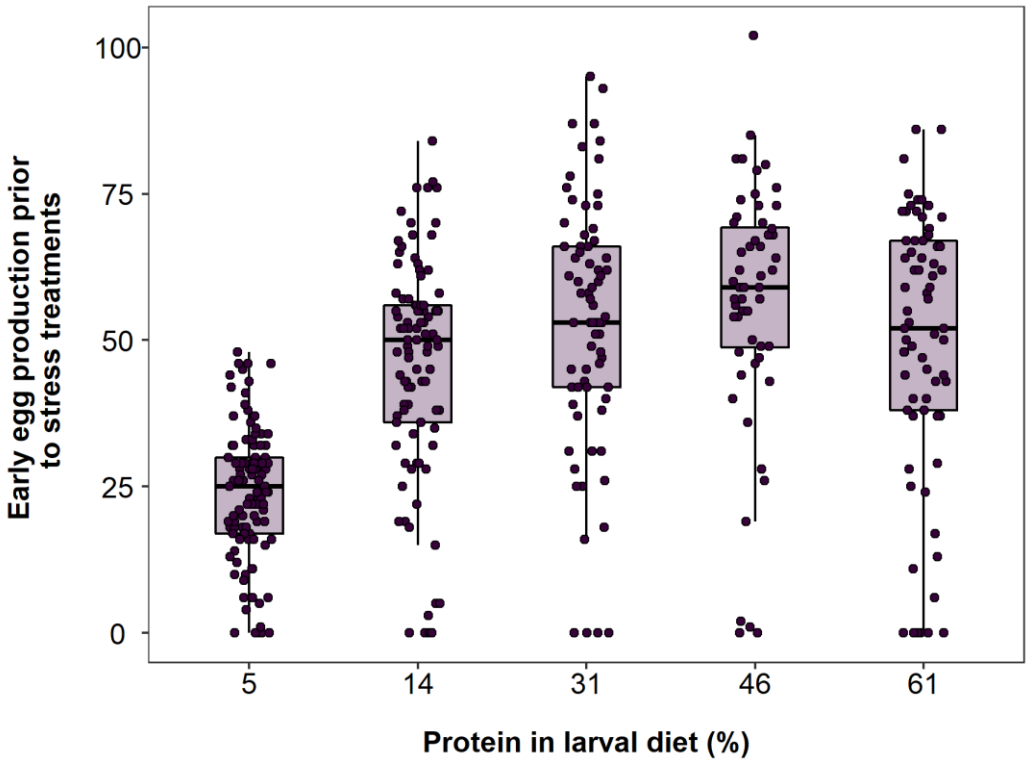

Figure 3.7: Effects of protein in larval diet on the number of eggs produced in the first week before stress treatments. The lines in the box plots indicate median values (50% quantile), boxes are the interquartile range (25% to 75% quantiles) and whiskers are minimum or maximum quartiles (25% - 1.5 x interquartile range, 75% + 1.5 x interquartile range).

Table 3.7: Model summary of a zero-inflated negative binomial model of the effects of protein in larval diet and stress treatments on the total number of eggs produced per fly in the first week. Block and individual ID are added as random effects. Protein protein^2 and lifespan are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded.

	Estimate	Standard error	Z value	Df	Chisq	Pr (>Chisq)
Intercept	3.97	0.08	48.60			
Protein	0.29	0.06	5.22	1	3.71	0.054
Protein^2	**-0.20**	**0.04**	**-4.77**	**1**	**22.19**	**<0.001**

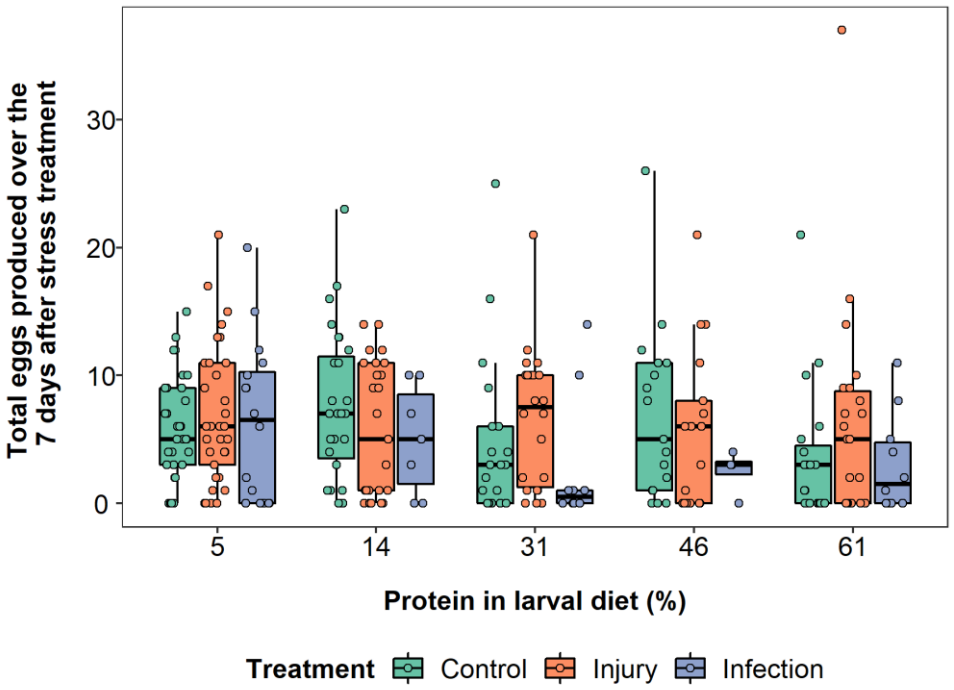

Figure 3.8: Effects of protein in larval diet on total eggs produced over seven days after stress treatment by flies infected with a bacterial pathogen (blue data points and bars), injured by a pinprick (orange data points and bars) or with no treatment (green data points and bars). The lines in the box plots indicate median values (50% quantile), boxes are the interquartile range (25% to 75% quantiles) and whiskers are minimum or maximum quartiles (25% - 1.5 x interquartile range, 75% + 1.5 x interquartile range).

Table 3.8: Summary of a negative binomial model analysing the effect of P:C in larval diet and stress treatments on the total number of eggs produced per fly seven days after stress treatments with (A) main effects parameter estimates and associated LRT test and (B) for the full model. Protein and protein^2 are mean centered to standard deviation of 1. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	Z value	Df	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:						
Injury treatment	0.12	0.12	0.99	2	2.36	0.31
Infection treatment	-0.13	0.18	-0.74			
Protein	-0.05	0.06	-0.76	1	0.59	0.44
Protein^2	0.003	0.08	0.04	1	0.002	0.97
(B) Full model parameter estimates and LRT test values for interactions:						
Intercept	2.08	0.14	14.37			
Injury treatment	-0.07	0.20	-0.35			
Infection treatment	-0.62	0.34	-1.79			
Protein	-0.02	0.10	-0.15			
Protein^2	0.14	0.13	-1.09			
Injury:Protein	0.06	0.14	0.43	2	5.57	0.06
Infection:Protein	-0.40	0.20	-1.97			
Injury:Protein^2	0.20	0.17	1.19	2	2.73	0.26
Infection:Protein^2	0.39	0.25	1.52			

The effect of larval P:C on lifetime egg production was similar to the effect on early-life reproduction, where increasing P:C in the larval diet increased lifetime egg production (Figure 3.3D & 3.9; Table 3.9; Protein = 0.11 (± 0.05), Chi-squared = 5.73, p = 0.02). The effect of P:C was non-linear (Figure 3.3D & 3.9;Table 3.9; Protein^2 = -0.11 (± 0.04), Chi-squared = 8.01, p = 0.005), with egg production reaching a peak at intermediate P:C and not increasing further at higher P:C. Stress treatment had a significant effect on lifetime egg production, where infected flies produced fewer eggs (Figure 3.3D & 3.9; Table 3.9; Treatment chi-squared = 8.81, p = 0.01; Infection = -0.18, (± 0.08); Injury = 0.04 (± 0.07)). Flies produced more eggs with longer lifespan (Table 3.9, Lifespan = 0.69 (± 0.04), Chi-squared = 275.77, p = <0.001). There was no significant interaction between larval P:C and stress treatment (Table 3.9).

A model not accounting for lifespan showed the same pattern with stress treatments having a significant effect on egg production, where infected individuals produced fewer eggs (Appendix C, Figure S3.13; Table S3.9; Stress treatment chi-squared = 80.68, p = <0.001; Infection = -0.82 (± 0.09); Injury = -0.04 (± 0.09)). Increasing larval P:C resulted in higher lifetime egg production, however this pattern was not quadratic (Appendix C, Figure S3.13; Table S3.9; Protein = 0.12 (± 0.04), Chi-squared = 5.91, p = 0.02; Protein^2 = - 0.07 (± 0.05), Chi squared = 2.10, p = 0.15). Even though the models differ slightly, the P:C patterns are broadly similar for both models, where egg production plateaus at the highest P:C level, as also seen in the raw data (Figure 3.3D & 3.9, Appendix C, Figure S3.13).

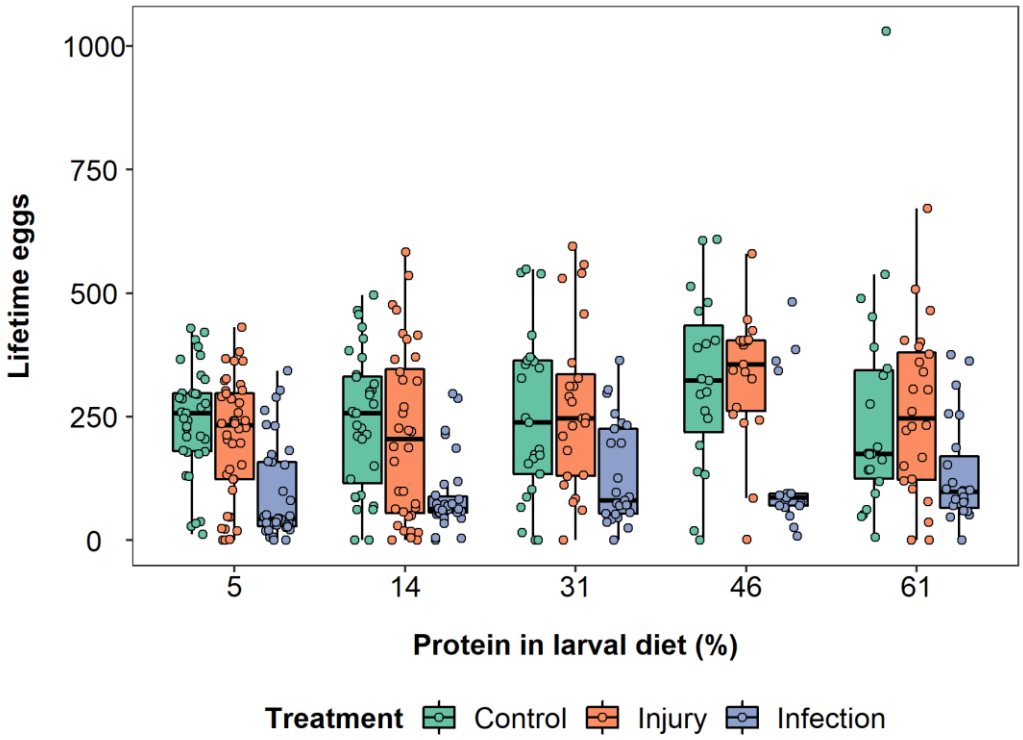

Figure 3.9: Effects of protein in larval diet on the lifetime eggs produced per female (up to day 98) of flies infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines). The lines in the box plots indicates median values (50% quantile), boxes are the interquartile range (25% to 75% quantiles) and whiskers are minimum or maximum quartiles (25% - 1.5 x interquartile range, 75% + 1.5 x interquartile range).

Table 3.9: Summary of a zero-inflated negative binomial model analysing the effect of P:C in larval diet and stress treatments on the on the total number of eggs produced per fly with lifespan added as a term in the model for (A) main effects parameter estimates and associated LRT tests and (B) for the full model. Protein, protein^2 and lifespan are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	Z value	Df	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:						
Injury treatment	**0.04**	**0.07**	**0.63**	**2**	**8.81**	**0.01**
Infection treatment	**-0.18**	**0.08**	**-2.30**			
Protein	**0.11**	**0.05**	**2.45**	**1**	**5.73**	**0.02**
Protein^2	**-0.11**	**0.04**	**-3.04**	**1**	**8.01**	**0.005**
(B) Full model parameter estimates and LRT test values for interactions:						
Intercept	5.27	0.08	64.23			
Injury treatment	0.06	0.11	0.54			
Infection treatment	-0.17	0.12	-1.41			
Protein	0.18	0.06	3.10			
Protein^2	-0.10	0.07	-1.60			
Lifespan	**0.69**	**0.04**	**19.78**	**1**	**275.77**	**<0.001**
Injury:Protein	-0.02	0.08	-0.29	2	2.39	0.30
Infection:Protein	0.10	0.08	1.18			
Injury:Protein^2	-0.02	0.09	-0.24	2	0.06	0.97
Infection:Protein^2	-0.008	0.09	-0.08			

In general, flies across all larval diets and stress treatments showed similar patterns in egg laying over their lifespan (Figure 3.3E & 3.10). Egg production was highest early in life and then declined (Figure 3.3E & 3.10). At mean centered P:C and lifespan, there was a slowing in the rate of decline of egg laying with age, such that the rate of decline is high when young and slows as flies get older (Table 3.10; Age = -0.59 (± 0.01), chi-squared = 2175.5, p = <0.001; Age^2 = -0.07 (± 0.01), chi-squared = 26.14, p = <0.001). As with lifetime egg production, there was a significant non-linear effect of P:C, with intermediate P:C diets resulting in higher daily egg production in the control stress treatment at mean age and lifespan (Figure 3.3E; Table 3.10; Protein^2 = -0.12 (± 0.03), chi-squared = 12.26, p = 0.0005). Lifespan had no effect on daily egg production (Table 3.10, Lifespan = 0.02 (± 0.02), p = 0.16).

The pattern of ageing in reproduction was broadly similar across larval diets and adult stress treatments (Figure 3.3E & 3.10). However, there were some significant two-, and three-way interactions, which indicate small differences in the pattern of reproductive ageing across diets and stress treatments. There was a significant two-way interaction between P:C and the linear and quadratic effect of age, suggesting that with higher P:C, ageing in egg production was quicker and the linear effect of age was highest at intermediate P:C diets (Figure 3.3E & 3.7; Table 3.10; Protein:Age = -0.07 (± 0.01), chi-squared = 30.48, p = <0.001; Protein:Age^2 = 0.07 (± 0.01), chi-squared = 23.94, p = <0.001). There was also a significant two-way interaction between age and the quadratic effect of P:C, suggesting that the rate of ageing was highest at intermediate P:C (Figure 3.32E; Table 3.10; Protein^2:Age = 0.07 (± 0.02), chi-squared = 22.62, p = <0.001). Stress treatments had significant effects on these two-way interactions (Figure 3.3E; Table 3.10, Treatment:Protein:Age^2 chi-squared = 14.41, p = 0.001, Treatment:Protein^2:Age chi-squared = 11.21, p = 0.004), where in injured flies these terms were smaller compared to the control individuals (Table 3.10; Injury:Protein:Age^2 = -0.08 (± 0.03); Injury:Protein^2:Age = -0.10 (± 0.03)). This suggests that there was less of an effect of P:C on aging in the injured flies. Stress treatment also had a significant

effect on ageing, where infected flies had a more negative decline in egg laying with age than control individuals (Figure 3.3E & 3.10; Table 3.10; Treatment:Age chi-squared = 18.05, p = <0.001; Infection:Age = -0.25 (± 0.06)).

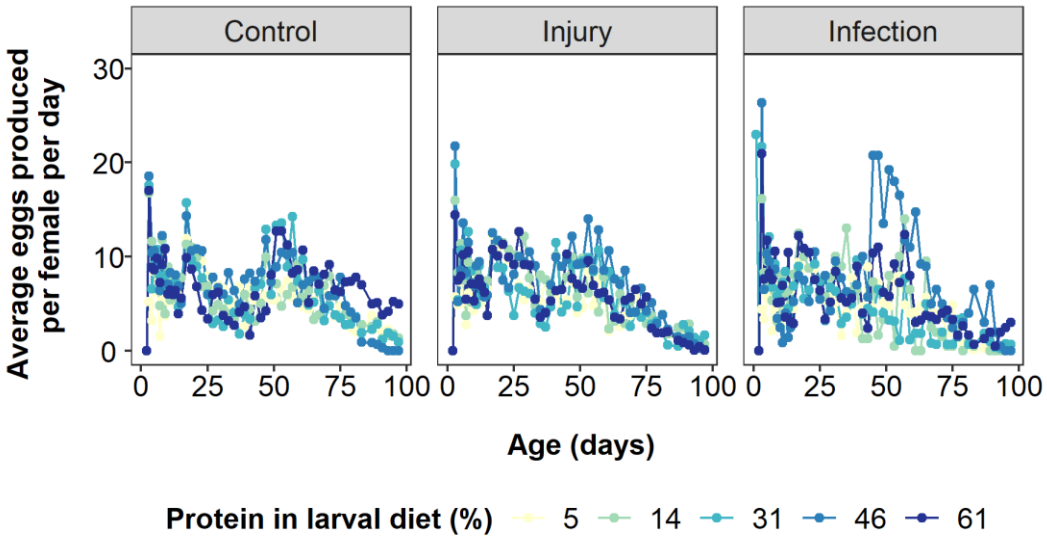

Figure 3.10: Average eggs per day for each stress and larval diet treatments on flies infected with a bacterial pathogen ("Infection"), injured by a pinprick ("Injury") or with no treatment ("Control"). For clarity, associated errors have been removed from the plot.

Table 3.10: Summary of a zero-inflated negative binomial model analysing the effect of P:C in larval diet and stress treatments on the daily number of eggs produced per fly (until day 98) for (A) main effects parameter estimates and associated LRT tests, (B) two-way interactions and (C) full model. Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded. Block and individual ID are added as random effects. (A, B) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	Z value	Df	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:						
Injury treatment	-0.02	0.04	-0.56	2	0.36	0.84
Infection treatment	-0.02	0.05	-0.44			
Protein	0.02	0.03	0.78	1	0.61	0.44
Protein^2	**-0.12**	**0.03**	**-4.05**	**1**	**12.26**	**0.0005**
Age	**-0.59**	**0.01**	**-47.22**	**1**	**2175.5**	**<0.001**
Age2	**-0.07**	**0.01**	**-5.13**	**1**	**26.14**	**<0.001**
(A) Two-way interaction parameter estimates and LRT test values:						
Protein:Age	**-0.07**	**0.01**	**-5.52**	**1**	**30.48**	**<0.001**
Protein:Age^2	**0.07**	**0.01**	**4.88**	**1**	**23.94**	**<0.001**
Protein^2:Age	**0.07**	**0.02**	**4.76**	**1**	**22.62**	**<0.001**
(C) Full model parameter estimates and LRT test values for interactions:						
Intercept	1.71	0.07	26.14			
Injury treatment	0.08	0.08	1.06			
Infection treatment	-0.09	0.10	-0.86			
Protein	0.02	0.05	0.41			
Protein^2	-0.05	0.05	-1.13			
Age	-0.60	0.03	-21.74			

Age^2	-0.03	0.02	-1.63			
Lifespan	0.02	0.02	1.41	1	2.00	0.16
Injury:Protein	0.07	0.06	1.17	2	1.61	0.45
Infection:Protein	0.002	0.07	0.03			
Injury:Protein^2	-0.09	0.06	-1.54	2	2.66	0.26
Infection:Protein^2	-0.01	0.07	-0.15			
Injury:Age	**-0.05**	**0.04**	**-1.18**	**2**	**18.05**	**<0.001**
Infection:Age	**-0.25**	**0.06**	**-4.27**			
Injury:Age^2	0.07	0.03	-2.30	2	5.90	0.052
Infection:Age^2	-0.06	0.04	-1.50			
Protein:Age	-0.10	0.02	-4.86			
Protein:Age^2	0.10	0.02	4.24			
Protein^2:Age	0.12	0.02	5.10			
Injury:Protein:Age	0.05	0.03	1.64	2	2.75	0.25
Infection:Protein:Age	0.03	0.04	0.82			
Injury:Protein:Age^2	**-0.08**	**0.03**	**-2.54**	**2**	**14.41**	**0.001**
Infection:Protein:Age^2	**0.08**	**0.04**	**1.75**			
Injury:Protein^2:Age	**-0.10**	**0.03**	**-3.18**	**2**	**11.21**	**0.004**
Infection:Protein^2:Age	**-0.003**	**0.05**	**-0.07**			

3.5 Discussion:

The main objective of our study was to test whether larval diets ranging in P:C content affected adult survival post-infection. We predicted that adults that developed on lower P:C diets would have worse survival post-infection, due to the demonstrated importance of dietary protein for the response to infection (e.g. Lee et al. 2006; Povey et al. 2009; Cotter et al. 2019; Chapter 2). However, our results provide no evidence for an effect of larval P:C diet on overall adult lifespan, regardless of stress treatment. Similarly, although intermediate P:C in the larval diet increased lifetime and early-life reproduction, there were no interactions between larval diet and stress treatment on reproduction, except for a smaller effect of P:C on the senescent decline in egg production for injured flies. Infection did however have effects on many life-history traits, specifically reducing lifespan and lifetime egg production, and increasing the rate of senescence in egg laying. These results suggest that, in this study, although P:C in larval diet affected larval and adult life-history traits, and exposure to infection in adulthood affected adult life-history traits, these effects did not interact very strongly.

Previous studies have suggested links between larval diet and the ability of adults to cope with environmental stress such as infection. For example, lower P:C nymphal diet with matching adult diet decreased adult survival post-infection measured for five days in female *Gryllus texensis* crickets (Kelly & Tawes, 2013). Similarly, adult *Anopheles gambiae* mosquitoes raised as larvae on increasing CR had lower melanising ability (Suwanchaichinda & Paskewitz, 1998). Studies measuring components of the immune system without a direct stressor have also shown that adults raised on non-optimal diets had lower adult immune function, with short term starvation (De Block & Stoks, 2008) or caloric restriction (Rolff et al., 2004). Similarly, adult *D. melanogaster* raised on higher P:C diets had higher levels of *Diptericin A* and *Metchnikowin* AMP transcription (Fellous & Lazzaro, 2010). This is particularly relevant to our study, as AMPs are important in bacterial defence (reviewed in Zhang and Gallo, 2016) and in *D. melanogaster* AMPs including *Metchnikowin* and *Diptericin* are upregulated with *P. entomophila*

infection (Liehl et al., 2006; Chakrabarti et al., 2012). However, in this previous study, only the amount of yeast in diet was altered without reducing carbohydrates thus altering both calorie and P:C content. Consequently, increased calories may be driving these effects and not the increase in P:C. As previous studies have all manipulated calories, perhaps the caloric value of juvenile diet affects adult immunity and not the macronutrient content. By only manipulating the P:C content of larval diets, our results suggest that macronutrient ratio does not affect adult survival post-infection.

Outside of differences in the type of diet manipulation, another factor that could explain our contrasting findings to previous research is the type of stress experienced. For example, *D. melanogaster* flies raised on higher P:C had a longer chill coma recovery time (Andersen et al., 2010) and worse starvation resistance (Davies et al., 2018), however better heat coma and desiccation resistance (Andersen et al., 2010). It has been suggested that these larval diet effects are a result of effects on general body condition or specific tissues (Fellous & Lazzaro, 2010), the production of heat shock proteins (Andersen et al., 2010), and more directly, lipid storage through eating a diet richer in carbohydrates as larvae (e.g. Roeder and Behmer, 2014; Kim, Jang and Lee, 2020). Our results suggest this does not seem true for immune responses, even though the fat body is an organ linked to both larval feeding and immune responses (reviewed in Arrese and Soulages, 2010). Larval feeding may therefore differentially affect adult stress response where certain diets are better for specific environmental stressors.

Another consideration is the timing of the stressor, as previous studies have applied stressors closer to eclosion. Our seven day lag post-eclosion could allow enough time for compensatory mechanisms (reviewed in Nestel *et al.*, 2016), such as compensatory feeding (Raubenheimer & Simpson, 1993) to mask any effects of larval diet. For example, adults that developed on low P:C diets could have eaten enough protein to survive injury and infection to a similar level to adults that developed on higher P:C. Two studies that altered both larval and adult diets found that adult environment was the main determinant of life-history traits (Davies et

al., 2018; Duxbury & Chapman, 2020), however, in one there were small and complex differences in female lifetime reproduction between larval and adult diet combinations (Duxbury & Chapman, 2020). It would therefore be interesting to repeat our experiment and expose adults to injury and infection stress immediately upon eclosion.

There was no effect of larval diet on survival or lifespan, as seen in other studies (Tu & Tatar, 2003; Houslay et al., 2015; Davies et al., 2018). In adults, altering P:C affects lifespan, with lifespan maximised on either intermediate (e.g. Lee 2015; Kim et al. 2020; Chapter 2) or low P:C diets (e.g. Lee *et al.*, 2008; Maklakov *et al.*, 2008; Jensen *et al.*, 2015). A small number of studies have suggested an effect of larval diet P:C on adult lifespan, but results have been inconsistent (lifespan maximised on high (Duxbury & Chapman, 2020), intermediate (Runagall-McNaull et al., 2015; Kim et al., 2019), and low (Economos & Lints, 1984; Stefana et al., 2017) P:C diets). A more consistent role has been suggested for calories, with adult lifespan decreasing with larval calorie restriction (May et al., 2015; Adler et al., 2016; Hooper et al., 2017; Krittika et al., 2019). Given a meta-analysis found no overall effect of early-life diet on lifespan (English & Uller, 2016), such contrasting findings suggest no clear effect of larval diet on adult lifespan and instead suggest that lifespan is more determined by adult diet.

Further analysis of lifespan data where individuals were categorised as dying close to the infection treatment or surviving this initial higher mortality, overall suggests that larval diet P:C has no strong effect on adult mortality. The sample sizes in many of the larval diet and stress treatments were very low, and these analyses should be interpreted with caution. In general, these analyses were similar to the full dataset analysis, however for the survival analysis of flies that survived the initial higher mortality, there was a significant interaction between larval diet in the infected flies. This may potentially suggest that low and high larval diets were detrimental to later-life survival in infected flies. There are several considerations to take into account with this result. First, out of the four analyses on this subset, only the Cox proportional hazards model had this significant effect.

Second, the assumption of proportional hazards was not met. Third, this effect may be due to the small number of infected flies surviving to this point, where the remaining infected flies may not be representative of a larger sample. Similarly, for the flies that died close to stress treatments, in the lifespan model, infected flies lived longer, again most likely due to small sample sizes of unstressed and injured flies dying in this time-period. Further experiments with higher sample sizes of flies in each category are required to test these patterns further. In addition, infection status of flies that survived to this later time point should be measured to determine whether the analysis is focusing on flies that are still infected or whether infection has been cleared, or whether the flies were never infected.

Lifetime and early-life reproduction increased with increasing larval P:C and then declined slightly at the highest P:C. Similarly, ovariole number has been show to peak at intermediate larval P:C in *Zaprionus indianus* (Matavelli et al., 2015) and in *Drosophila melanogaster* (Rodrigues et al., 2015). Many larval studies lack this decline at the highest P:C diets (e.g. Tu and Tatar, 2003; Andersen *et al.*, 2010; Silva-Soares *et al.*, 2017; Duxbury and Chapman, 2019; Kim *et al.*, 2019). Protein is often the limiting nutrient in egg production (reviewed in Wheeler, 1996; Boggs, 2009), but can have a toxic effect when consumed at very high levels (reviewed in Simpson and Raubenheimer, 2009), which may explain the plateauing at very high P:C due to larvae of worse condition developing to adults. Due to the highest P:C ratio also including the lowest carbohydrate content, this effect may also be due to a limiting effect of carbohydrates on development. As nutrients for egg production can be acquired from adult feeding and nutrient requirements can differ between species (reviewed in Wheeler, 1996), this limit may not always appear. These P:C effects could also arise through the general increase in body condition with higher P:C in larval diet (Runagall-McNaull et al., 2015). Overall, there appears to be an increase in adult reproduction with increasing P:C in the larval diet, but this effect may plateau at very high P:C levels.

Infection reduced lifetime egg production, a typical response in insects (reviewed in Schwenke et al. 2016), however there was no interaction between

larval P:C and reproduction post-infection. When accounting for the overall shorter lifespan of infected flies, their reproduction was comparable to the injured or unstressed flies. In the week after stress treatments, all treatment groups produced the same number of eggs, however for the infected group the number of reproducing individuals was lower compared to control or injured flies. This suggests that with infection, individuals were able to produce more eggs earlier in life but then egg numbers declined. This was reflected in the reproductive ageing results, as infection increased the rate of senescence in egg laying. This could be evidence of terminal investment (Clutton-Brock, 1984), specifically fecundity compensation, as flies shifted their egg production earlier as a response to infection, as is a common outcome after infection (reviewed in Kutzer and Armitage, 2016).

In general, the patterns of reproductive ageing were quite similar across treatments, but there were some interactions between P:C in the larval diet and stress treatment. Overall, at intermediate P:C, ageing in egg production was quickest. Similar results in reproductive ageing have been found in studies altering adult P:C (e.g. Jensen et al. 2015; Chapter 2). These results are also similar to previous studies focusing on ageing in egg laying where adults raised on higher P:C and/or calories as larvae appear to have quicker ageing in egg laying (Tu and Tatar, 2003; Hooper *et al.*, 2017, but see May, Doroszuk and Zwaan, 2015). As these studies manipulated calories, here we show there are minor changes in ageing patterns also with P:C manipulation of the larval diet.

For the larval traits, we predicted that larvae would have more successful and quicker development on higher P:C diets (Britton & Edgar, 1998; Colombani et al., 2003; Chang, 2004; Mirth & Riddiford, 2007). Development time was quickest on intermediate P:C, and egg-to-pupae and egg-to-adult viability were higher on higher P:C, as seen in previous studies (e.g. Andersen et al. 2010; Silva-Soares et al. 2017; Kutz et al. 2019, but see Houslay *et al.*, 2015; Davies *et al.*, 2018; Gray, Simpson and Polak, 2018). However, despite being statistically different, the effect of diet on egg-to-pupae and egg-to-adult viability was small. There was no effect of

P:C on pupae-to-adult viability, suggesting that all of the diets used in our study allowed larvae to pupate successfully. Studies often do not report pupae-to-adult viability, however more extreme diets could affect this trait as different sources of carbohydrates have been shown to affect pupae-to-adult viability (Nash & Chapman, 2014). On the highest P:C diet, development time was slightly slower, which is also a common finding (e.g. Lee et al. 2012; Rodrigues et al. 2015; Kutz et al. 2019), again potentially due to toxic effects of high P:C diets (reviewed in Simpson and Raubenheimer, 2009) or due to a limitation of carbohydrates for development. Vials with more eggs took longer to develop, most likely due to larval density effects (e.g. Ludewig *et al.*, 2017; Klepsatel, Procházka and Gáliková, 2018; Henry, Tarapacki and Colinet, 2020; Than, Ponton and Morimoto, 2020). Our work adds to growing evidence of the importance of macronutrients in larval diet for larval development and suggest that, with some exceptions, intermediate P:C diets are better for key larval traits.

3.6 Conclusions and future work:

The results of this study suggest that larval dietary P:C has no effect on adult survival with or without stress treatment, and thus larval P:C does not alter the long-term consequences of injury or infection on survival. Intermediate P:C larval diets were optimal for many traits pre-, and post-metamorphosis. Individuals were the quickest to develop into adults on intermediate larval P:C, and subsequent adults produced the most early-life and lifetime eggs. Larvae were more likely to develop into adults on higher P:C. Therefore, our results add to the growing evidence that larval diet affects adult life-history traits, but the long-term consequences of infection and injury are not altered. To understand the effects of larval diet on the ability of adults to respond to infection further, we suggest experiments exposing adults to infection immediately after eclosion to avoid potential for compensatory feeding. Furthermore, using a fully factorial experiment combining variation in larval and adult diet could help to disentangle the differences between larval and adult feeding on stress responses such as infection and injury.

Chapter 4:

No evidence of changes in short-term food choice following infection in *Drosophila melanogaster*

4.1 Abstract:

According to the optimal foraging theory, individuals should choose diets that maximise their fitness. Across taxa, food choice is driven by multiple factors including availability of foods, and internal and reproductive state. Infection directly affects both fitness and often diet choice, and a common sickness behaviour is to limit food intake post-infection. Changing dietary protein to carbohydrate (P:C) ratios affect infection outcomes and infected individuals often alter diet preference to a P:C that improves post-infection survival. Using an outbred population of female fruit flies (*Drosophila melanogaster*) we tested whether systemic infection with a bacterial pathogen (*Pseudomonas entomophila)* alters food preference when individuals are offered a choice between two diets: a pure carbohydrate diet (0:1 protein to carbohydrate, P:C, 0% protein) or a diet also containing protein (1:4 P:C, 20% protein). In a previous experiment using the same host-pathogen pair, infected and uninfected individuals had higher reproduction and survival on higher P:C diets. The increase in survival was much greater for infected individuals as they had very low survival on low P:C diets. Therefore, we predicted that infected individuals would show greater preference for the diet containing protein, and in line with a common sickness behaviour, would consume less food. Surprisingly, infection had no effect on food choice tested in the short-term, as infected flies consumed the same amount of food and showed no altered preference compared to the injured and uninfected flies. All individuals chose the higher P:C diet, however this was only observed above a certain threshold of total sips. These results suggest that *D. melanogaster* do not alter their short-term food choice post-infection with *P. entomophila*, despite potential fitness benefits for the host of consuming a higher P:C diet.

4.2 Introduction:

Food choice is a complex behavioural trait, where individuals choose beneficial and avoid potentially toxic diets (reviewed in Yarmolinsky et al., 2009; Sepil et al., 2020). Food choice behaviour is under neuronal control, where internal state (e.g. reproductive state and individual nutritional history), nutrient evaluation and food availability influence feeding (reviewed in Münch et al., 2020). Another factor that may affect food choice is infection, and reduced food intake is a common sickness behaviour (reviewed in Hite et al., 2020). Importantly, the composition of the diet also influences infection outcomes (reviewed in Ponton et al., 2011b, 2013). When infected individuals are given a food choice, they often select diets that increase survival post-infection (e.g. Lee et al., 2006; Povey et al., 2009; Dinh et al., 2019; Ponton et al., 2020). Following on from an experiment demonstrating an effect of diet on the outcome of infection in *Drosophila melanogaster* with the bacterial pathogen *Pseudomonas entomophila* (Chapter 2), here we test whether infection with *P. entomophila* alters food choice measured in the short-term.

Previous food choice tests have manipulated diets in many different ways, and as predicted by optimal foraging theory, generally individuals choose diets that maximise fitness (Pyke et al., 1977; Stephens & Krebs, 1986; Pyke, 2019). One particular area of interest has been the manipulation of macronutrients, often through altering protein to non-protein ratios (reviewed in Simpson et al., 2004). Such manipulations have allowed for estimations of intakes of various macronutrients at which fitness is maximised, which are called intake targets (reviewed in Simpson et al., 2004). Studies combining food choice and measures of multiple life-history traits often show that individuals choose P:C diets that correspond with higher lifetime reproductive success (Lee et al. 2008; Fanson et al. 2009; Jensen et al. 2012, but see Maklakov et al. 2008; Fanson et al. 2009; Harrison et al. 2014; Bunning et al. 2016). For example, when offered various binary food choices, *D. melanogaster* have been found to converge on an intake of ~1:4 P:C (Lee et al., 2008; Jensen et al., 2015; Ponton et al., 2020). This intake ratio is close to the

diet that maximises lifetime reproductive success (Lee et al., 2008; Jensen et al., 2015).

Intake targets are not fixed points and various costs associated with activities such as flying and environmental variables such as temperature or infection may alter intake requirements, and therefore intake targets (reviewed in Simpson & Raubenheimer, 2012). For example, infection alters the diet that is more optimal for survival measures (Lee et al. 2006; Povey et al. 2009; Chapter 2). As optimal P:C diets have been found to vary with infection, food choice for infected individuals may also differ (reviewed in Simpson & Raubenheimer, 2012).

A commonly observed sickness behaviour is a decrease in food intake (reviewed in Kyriazakis et al. 1998; Hite et al. 2020, see Table 1 in Sullivan et al. 2016). A decrease in food intake has also been recorded in insects (e.g. Ayres and Schneider 2009; Singer et al. 2014, but see Tyler et al. 2006; Köhler et al. 2012). Furthermore, forcing individuals to eat more when infected can have negative consequences (Murray & Murray, 1979; Sullivan et al., 2016). For example, linseed oil force-fed *Manduca sexta* caterpillars had decreased survival post-infection compared to force-fed uninfected caterpillars, although the same patterns were not apparent with forced feeding of sucrose or water (Sullivan et al., 2016). This decreased feeding response is somewhat paradoxical, as responding to infection requires both energy and nutrients (reviewed in Lochmiller & Deerenberg, 2000). Various hypotheses for this behaviour have been proposed, including that limiting feeding may starve the pathogen, promote the host immune response, or that the host is more picky in the foods they choose once infected (e.g. Hart, 1988; Kyriazakis et al., 1998). This decrease in feeding could also be due to infected individuals attempting to limit intake of a specific macronutrient to optimise immune function (Ponton et al., 2020; Hite et al., 2020).

Infection status can also affect food choice, as infected individuals prefer diets of different macronutrient compositions (e.g. Lee et al., 2006; Povey et al., 2014; Ponton et al., 2020; Hite et al., 2020; Sieksmeyer et al., 2021). As with the above, these studies often find that overall feeding reduces with infection even

when offered a choice between foods containing different P:C ratios (Lee et al., 2006; Mason et al., 2014; Povey et al., 2014; Dinh et al., 2019; Ponton et al., 2020; Sieksmeyer et al., 2021). However, in some experiments, food intake increased (Povey et al., 2009; Shikano & Cory, 2016). Some of this inconsistency may be due to pathogen specificity, with some pathogens not resulting in a reduction in food intake (Ayres & Schneider, 2009; Shikano & Cory, 2016).

Independent of a reduction in food intake, there is also evidence for an effect of infection on the preferred dietary macronutrient composition, with individuals either preferring higher (Lee et al., 2006; Povey et al., 2014; Shikano & Cory, 2016; Sieksmeyer et al., 2021) or lower P:C ratios (Mason et al., 2014; Dinh et al., 2019; Ponton et al., 2020). These differences appear to reflect which diets are beneficial for surviving infection in each host-pathogen system (Lee et al. 2006; Povey et al. 2014; Dinh et al. 2019; Ponton et al. 2020, but see Sieksmeyer et al. 2019). In no-choice tests where individuals were fed the preferred P:C diet, many measures of the immune response were higher (Lee et al., 2006; Mason et al., 2014; Povey et al., 2014; Ponton et al., 2020). In one study measuring pathogen loads post-infection, individuals had a lower pathogen load a few days post-infection on the preferred diet (Dinh et al., 2019). Overall, there is evidence for considerable host-pathogen specificity in diet choice following infection, but individuals generally appear to choose diets that have a survival benefit post-infection.

Here we test whether food choice, measured in the short-term (intake targets were not measured), changes with infection using female *D. melanogaster* and the bacterial pathogen *P. entomophila*. Food choice in *D. melanogaster* is a multifaceted trait combining multiple cues, including visual, olfactory and chemosensory cues (reviewed in Kaushik & Kain, 2020). Previous *D. melanogaster* studies have demonstrated clear preferences based on variation in P:C content (Lee et al., 2008; Jensen et al., 2015). In a previous study using the same host-pathogen pair, uninfected and infected flies all had improved survival and reproduction as the P:C of the diet increased from very low levels (Chapter 2). This difference in survival with increasing P:C was much greater in the infected flies, as

they had very low survival on low P:C diets (Chapter 2). In the food choice test, we offered flies the choice between a diet that contained only carbohydrate (0:1 P:C, 0% protein) and one with carbohydrate and protein (1:4 P:C, 20% protein). We predicted that all flies would choose the diet with higher P:C, however this would be more pronounced in the infected flies as the improvement in survival was much greater with infection. When larvae are orally infected with *P. entomophila*, they stop feeding due to a food uptake blockage (Vodovar et al., 2005; Liehl et al., 2006). To our knowledge, this has not been tested in *D. melanogaster* adults. However, with *P. entomophila* infection in *Blatta orientalis* cockroaches, individuals did not fully stop feeding, but showed a decrease in overall feeding and preferred a higher P:C ratio post-infection (Sieksmeyer et al., 2021). If food blockage post-infection with *P. entomophila* occurs in both *D. melanogaster* larvae and adults, we expected to see no feeding in infected individuals.

4.3 Methods:

4.3.1 Experimental flies:

The *D. melanogaster* used in the food choice experiment were from the eggs of the 60th generation of the same outcrossed *Drosophila melanogaster* genetic reference panel (DGRP) population as described elsewhere (Chapter 1, section 1.11.1, and Appendix A). We pipetted 5 µl of egg solution into each of 20 vials (following Clancy and Kennington 2001). Flies were housed at 25°C at 12:12 L:D light cycle on modified Lewis food (Lewis 1960, see 1:6 P:C (14% protein) diet in Table 1.1, Chapter 1, section 1.11.2). Using an outcrossed population from these lines should produce a population with standing genetic variation in food choice, as the individual DGRP lines show genetic variation in food choice (Toshima et al., 2014).

To generate experimental individuals, ancestral flies were tipped under controlled density for two generations prior to collection. The F0 generation was tipped with a density of 10 females to 2 males into each of five vials and the F1 generation with a density of 5 females to 2 males. To ensure F2 experimental flies were aged matched, freshly eclosed F2 flies were collected on the 12th day after the F1 generation was established. In total, there were 6 blocks of experimental fly collection, with each block following the procedure outlined above. Experimental individuals were left in mixed-sex age-matched groups until seven-days-old, following which females were collected and infection treatment applied (see below). By maintaining flies in mixed-sex groups for 7 days, we ensured that all females were mated and thereby had the same mating status, as mating status in females alters food choice (e.g. Ribeiro & Dickson, 2010).

4.3.2 Infection treatments:

On the seventh day post-eclosion for each block, female flies were treated with one of three treatments as described elsewhere (Chapter 1, section 1.11.3 and Chapter 3, section 3.3.4). An injury treatment was included to test whether the septic infection method by itself without an infective pathogen changed food

choice. Untreated flies were briefly anaesthetised under CO_2 to match the other treatments, as CO_2 treatment is known to affect some behavioural measures (Bartholomew et al., 2015; MacMillan et al., 2017).

The re-suspended overnight culture was grown for about four hours until it reached an optical density (OD) of at least 0.2, and then diluted over 100 times to match a dilution of 0.001 OD, as described previously (see Chapter 1, section 1.11.3 and Chapter 3, section 3.3.4). To minimise time-of-day effects on immunity (e.g. Lee & Edery, 2008) and to test food choice in individuals after a similar time post-infection, infection treatments were done at the same time each day (13:00). After infection treatments, the flies were wet starved (put in empty vials with slightly wet cotton ball closing the vial and wet paper at the bottom of the vial) for 21 hours prior to the food choice assays (see below). Per treatment per block, 30 flies were treated and housed across two vials (15 flies in each). To confirm bacterial establishment within the fly and to quantify bacterial load, we plated all the flies used in the food choice assays on *Pseudomonas* isolating agar and counted resulting colony forming units (CFUs, following Gupta et al. 2017, see Chapter 1, section 1.11.4).

4.3.3 Food choice assay (flyPAD):

The food choice assay was carried out using an automated system called the fly Proboscis and Activity Detector (flyPAD) (Itskov et al., 2014). The flyPAD works by detecting and recording a change in electrical capacitance every time a fly proboscis touches a dried droplet of medium placed on an electrode within a circular arena (hereafter referred to as a "sip"). This contact may also be due to a fly leg touching the food, however from a manual video analysis, the number of registered sips correlates well with proboscis extension (Itskov et al., 2014). The food choice assay in each arena consisted of two diets, each placed on an individual electrode: (1) "0:1 P:C" with 10% sucrose in 2% agar (0% protein), and (2) "1:4 P:C" with 8% sucrose and 2% yeast in 2% agar (20% protein) (see Appendix D, Figure S4.1). For the 1:4 P:C value, yeast is considered to be fully composed of protein, however this P:C ratio would be closer to 1:8.8 P:C with an estimation that

Fermipan Red yeast as 50% protein, 40% carbohydrate (Turner Price, 2021). These amounts were chosen to keep the diets isocaloric with only the amount of yeast and sucrose differing between them (Mair et al., 2005). Pilot data (not shown) suggested the 1:4 P:C ratio was the highest P:C ratio that could be used before food became unpalatable and flies stopped feeding. Therefore, multiple diet pairs and higher P:C ratios could not be used. To make the diets, the total food ingredients adding up to 1 g were mixed in 10 ml distilled water with 0.2 g agarose in a water bath at 70°C (modified from Itskov et al. 2014). This solution was then stored in 200 µl Eppendorfs at -20°C and individual Eppendorfs were reheated to around 40°C before flyPAD experiments. A fresh aliquot was used per block.

To give each fly a choice between the two diets, 1 µl of each food type was pipetted onto each flyPAD electrode with two electrodes per arena (see Appendix D, Figure S4.1). To prevent any potential variation between electrodes, the two diets were pipetted on alternating sensors for each block. Food was left to dry prior to introducing flies to the arenas. Ten flies per treatment were individually placed in 30 flyPAD arenas (see Appendix D, Figure S4.1). This resulted in a final sample size of 60 flies per treatment (180 flies in total from the three infection treatments). The number of sips of each food flies took was recorded for an hour, with each block started at the same time of day to avoid behavioural time-of-day effects (10:00, 21 hours post-infection). To assess if there was any preference in the food chosen by flies, a commonly used measure of preference index was used (e.g. Itskov et al., 2014; Steck et al., 2018). Preference index shows which food is eaten more, where -1 and 1 indicate full preference for either food and 0 indicates no preference. The preference index was calculated as follows, where 1 indicates preference for 1:4 P:C and -1 for 0:1 P:C:

$$Preference\ index = \frac{1{:}4\ P{:}C\ sips - 0{:}1\mathrm{P}{:}\mathrm{C}\ sips}{Total\ sips}$$

4.3.4 Statistical methods:

The data were analysed using R software, version 4.0.2 (R Core Team 2014). All graphs were drawn using ggplot2 (Wickham, 2016). Flies that ate neither diet

were censored from the analysis (final sample sizes untreated control = 48, injury = 52, and infection = 55 per treatment from original 60) and there was no difference between treatments on whether a fly ate or not (Pearson's Chi-squared test, χ^2= 3.44, df = 2, p = 0.18). Flies which ate only one of the diets were included, as analysing the data without these data points made no difference to the results of the analysis (data not shown). Models included treatment as a categorical fixed effect and block as a random effect. Models were checked for zero inflation and overdispersion using the *DHARMa* package (Hartig, 2021). Prediction plots were made with all random effects set to 1.

The total number of sips taken was analysed using a generalized linear mixed model (GLMM) with a negative binomial distribution with the *glmmTMB* R package (Brooks et al., 2017). Preference index was analysed with a linear mixed model (LMM) using the lme4 R package (Bates et al., 2015). As there was considerable variation in the total sips taken across the dataset (total sips ranging from 1 to 2166) and preference index analysis is based on a proportion of the total number of sips, we further analysed how the total number of sips affected the number of 1:4 P:C sips taken. We used a LMM with total sips taken as a fixed effect and sips of 1:4 P:C diet as the response variable. Here, a linear slope of 0.5 would indicate that individuals with any given total sips took equal numbers of both diets. A slope closer to 1 would indicate that individuals which had more total sips took more sips of the yeast food, and a slope closer to 0 would indicate individuals which had more total sips took more sips of the 0:1 P:C diet. To analyse whether this relationship between a fly's total sips to sips of 1:4 P:C changed with injury or infection, an interaction term between total sips and treatment was added. For displaying the summaries of LRT results of the main effects, parameter estimates and associated standard deviations are from a separate model not including the total sips to treatment interaction.

A high proportion of infected flies had no quantifiable bacterial growth present (21/53 flies, about 40% of infected flies, with two infected flies removed from the analysis due to surface contamination). To see whether this variation

changed food choice in infected flies, we analysed the effect of bacterial growth presence as a binomial variable (0 for no bacterial growth, 1 for presence of bacterial growth) on total sips taken with a negative binomial model and on preference index with a LMM.

As analysing preference index required the use of linear models but preference index is bounded at +/-1, our results were confirmed with appropriate non-parametric tests (see Appendix D). As the analysis of the number of 1:4 P:C sips taken required a linear model to determine the number of sips taken on a linear scale for interpretation of the slope an intercept on the data scale, the statistical significance of the model was confirmed with a zero-inflated negative binomial model with total sips mean centered (see Appendix D, Table S4.1). As other aspects of the microstructure of meals can differ than just the number of sips (reviewed in Itskov et al., 2014; Münch et al., 2020), we analysed other measures from the flyPAD analysis, including more detailed information on sips, feeding bursts and activity bouts. They showed qualitatively similar patterns to the analysis presented and are not shown.

4.4 Results:

Regardless of injury or infection treatment, flies took the same number of total sips (Figure 4.1A; Table 4.1A; Treatment $\chi^2 = 5.06$, $p = 0.08$) and showed no preference for either the diet containing only sugar (0:1 P:C, 0% protein) or the diet containing sugar and a protein source (1:4 P:C, 20% protein) (Figure 4.1B; Table 4.1B; Treatment $\chi^2 = 1.50$, $p = 0.47$).

Individuals took very different numbers of sips in total, ranging from 1 to 2166 sips per fly, and there was a trend of flies above a certain threshold of total sips mostly choosing more of the 1:4 P:C diet (20% protein) (Figure 4.1A & 4.1C). By analysing how total sips taken by individual flies affected how many sips of the 1:4 P:C diet (20% protein) they took, flies with higher number of total sips took more sips of the 1:4 P:C diet (20% protein) (Figure 4.2, Table 4.2; Total sips = 0.90 ($\pm$ 0.02) $\chi^2 = 355.73$, $p = <0.001$). Here, a slope of 0.5 would mean that at any given total sips, flies took equal sips of both diets (see dotted 0.5 slope line in Figure 4.2). Therefore, a slope of 0.9 suggests that individuals that took a higher number of sips in total took more sips of the 1:4 P:C diet (20% protein). There was a threshold level of approximately 150 total sip above which flies took more sips of the 1:4 P:C diet (20% protein) (Figure 4.2). This suggests that individuals had a preference for the 1:4 P:C diet (20% protein), however they needed to have eaten enough sips in total prior to showing this preference. Infection treatment had no effect on this relationship, with infected, injured and control flies eating similar amounts of the 1:4 P:C diet (20% protein) with higher sips in total (Figure 4.2; Table 4.2; Treatment $\chi^2 = 0.29$, $p = 0.86$; Infection = -1.79 ($\pm$ 20.73); Injury = 8.15 ($\pm$ 20.91); Total sips:Treatment $\chi^2 = 1.91$, $p = 0.39$).

For the infected flies, individuals with a quantifiable bacterial growth (32/53, about 60% of flies) had an average of 12.83 ($\pm$ 3.83) $\log_2$ CFUs/ml. This variation in quantifiable infection load (or infection status) had no effect on food choice in the infected flies, as flies with or without quantifiable bacterial growth took similar numbers of total sips (Figure 4.3A & 4.3B; Table 4.3A; Bacterial growth $\chi^2 = 0.0007$,

p = 0.98) and showed no preference for either diet (Figure 4.3C & 4.3D; Table 4.3B,; Bacterial growth $\chi^2 = 0.42$, p = 0.52).

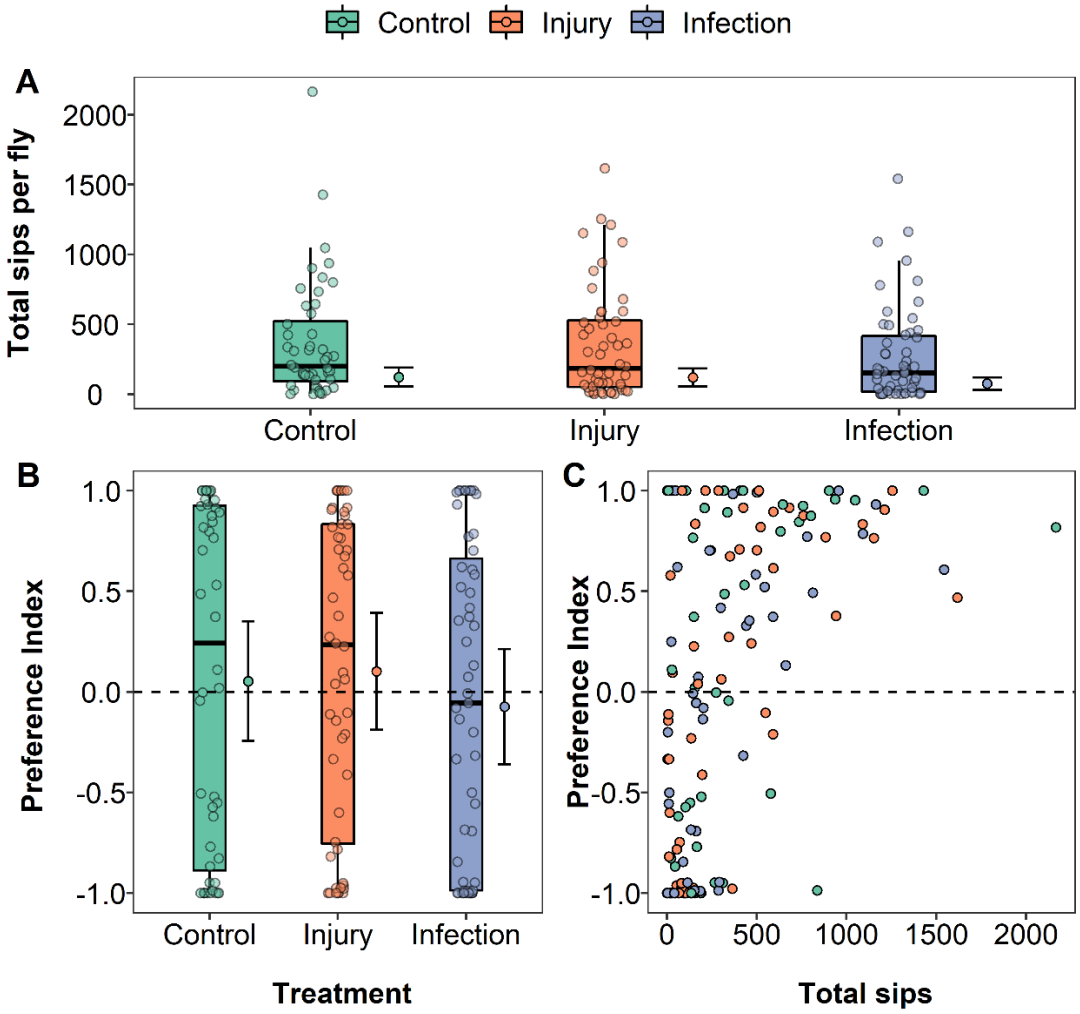

Figure 4.1: Effects of treatment on (A) total number of sips, (B) preference index, and (C) preference index with total sips, for flies infected with a bacterial pathogen (blue bars and data points), injured by a pinprick (orange bars and data points) or with no treatment (green bars and data points). (A, B) The lines in the box plots indicate median values (50% quantile), boxes are the interquartile range (25% to 75% quantiles) and whiskers are minimum or maximum quartiles (25% - 1.5 x interquartile range, 75% + 1.5 x interquartile range). (B, C) For preference index, 1 indicates preference for the 1:4 P:C food (20% protein), 0 for no preference (dotted line) and -1 for preference for the 0:1 P:C food (0% protein). A secondary point and associated error bars show (A) negative binomial model or (B) linear mixed effects model predictions, where error bars are 95% confidence intervals.

Table 4.1: Model summaries for the effect of treatment on (A) total number of sips or (B) food preference index. (A) Model summaries of models analysing total sips is a negative binomial model, and (B) model analysing preference index, where 1 indicates preference for the 1:4 P:C food, 0 for no preference and -1 for preference for the 0:1 P:C food, are from linear mixed effects models. Block was fitted as a random effect.

(A) Treatment effects on total number of sips:						
	Estimate	Standard error	Z value	Df	Chisq	Pr (>Chisq)
Intercept	5.68	0.34	16.55			
Injury treatment	-0.03	0.24	-0.12	2	5.06	0.08
Infection treatment	-0.48	0.23	-2.04			
(B) Treatment effects on food preference index:						
	Estimate	Standard error	T value	Df	Chisq	Pr (>Chisq)
Intercept	0.05	0.15	0.35			
Injury treatment	0.05	0.15	0.32	2	1.50	0.47
Infection treatment	-0.13	0.15	-0.84			

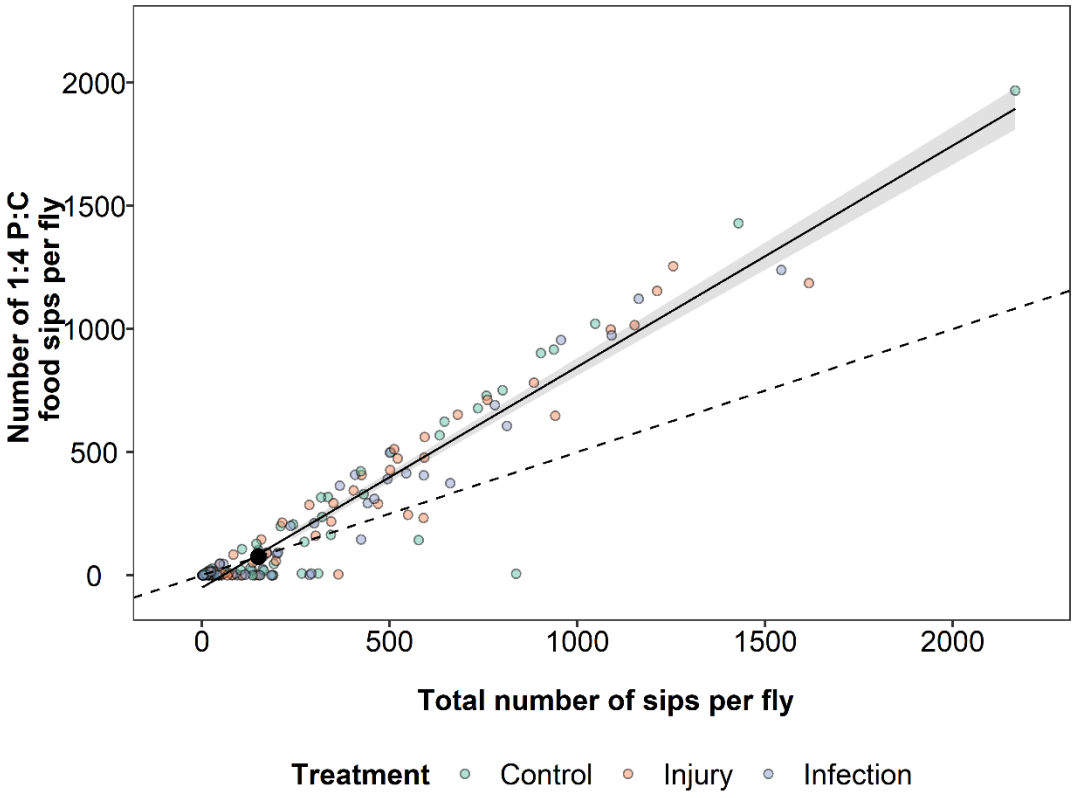

Figure 4.2: Effects of treatment and the total number of sips on the number of 1:4 P:C diet sips of flies infected with a bacterial pathogen (blue data points), injured by a pinprick (orange data points) or with no treatment (green data points). The dotted line shows where with any given total sips, flies had equal numbers of both diets (slope of 0.5). The black line shows model predictions for a model with no treatment included, where error bars are 95% confidence intervals. The black dot on this prediction line shows point at which model predicts that half of total sips were of 1:4 P:C (20% protein) at 150 total sips, where flies taking lower number of total sips preferred the 0:1 P:C (0% protein) sips and flies taking a higher number of total sips preferred the 1:4 P:C sips (20% protein).

Table 4.2: Summary of a linear mixed model of the effects of treatment and total sips taken on the number of 1:4 P:C sips taken with (A) main effects parameter estimates and associated LRT tests and (B) for the full model. Significant results below significance level α = 0.05 are bolded. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	T value	Df	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:						
Intercept	-52.02	17.79	-2.93			
Total sips	**0.90**	**0.02**	**39.40**	**1**	**355.73**	**<0.001**
Injury treatment	8.15	20.91	0.39	2	0.29	0.86
Infection treatment	-1.79	20.73	-0.09			
(B) Full model parameter estimates and LRT test values for interaction:						
Intercept	-66.46	20.86	-3.19			
Total sips	0.94	0.04	25.82			
Injury treatment	31.35	28.01	1.12			
Infection treatment	19.80	26.86	0.73			
Total sips:Injury treatment	-0.06	0.05	-1.25	2	1.91	0.39
Total sips:Infection treatment	-0.07	0.06	-1.20			

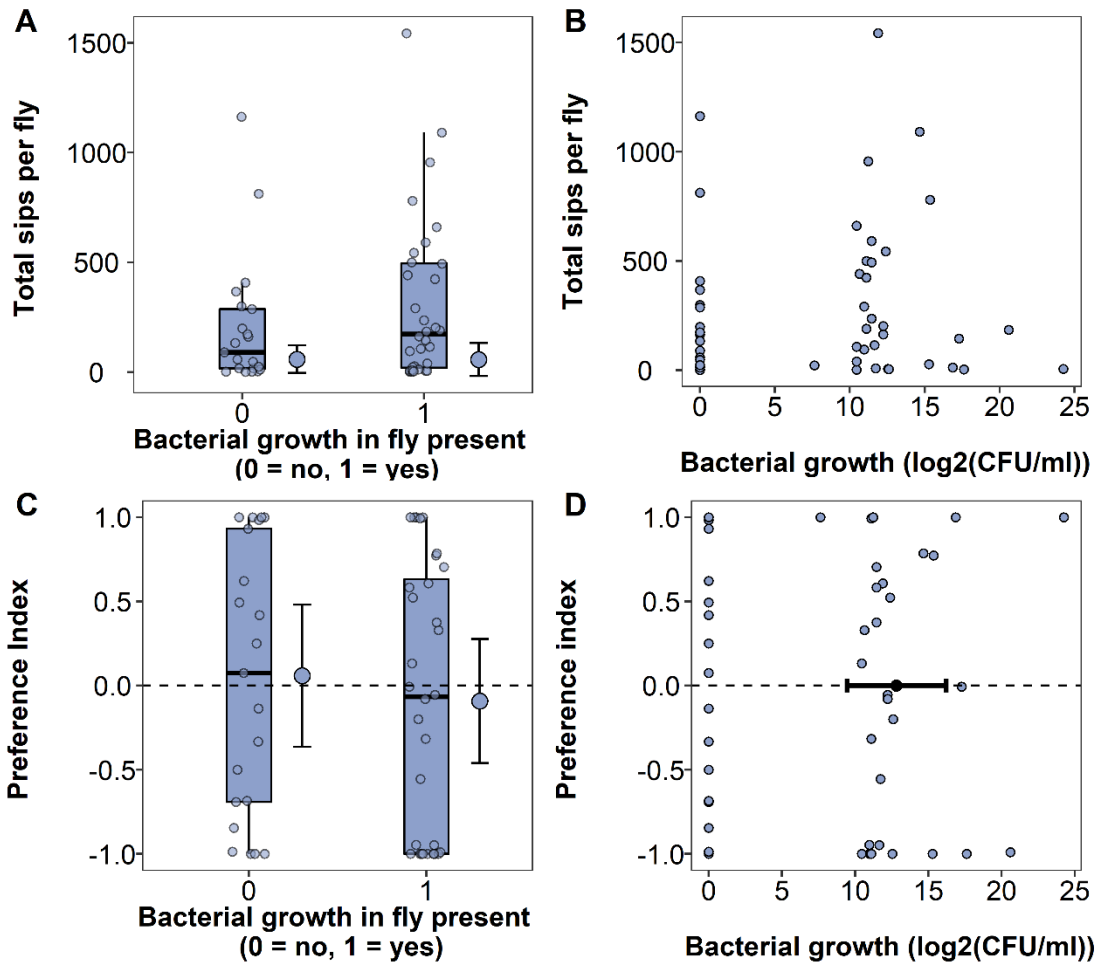

Figure 4.3: Effects of bacterial growth on (A, B) total sips taken and (C, D) on food preference index of infected flies, where 1 is preference for the 1:4 S:Y food, 0 is no preference (dotted line) and -1 is preference for the 0:1 S:Y food. Data is shown as either (A, C) a binomial variable, where 0 indicates no presence, 1 indicates presence of bacterial growth, or (B, D) as a continuous variable as CFU/ml (log_2 transformed). The lines in the box plots indicates median values (50% quantile), boxes are the interquartile range (25% to 75% quantiles) and whiskers are minimum or maximum quartiles (25% - 1.5 x interquartile range, 75% + 1.5 x interquartile range) (A, C). A secondary point and associated error bars next to boxplots show (A) negative binomial model or (C) linear mixed effects model predictions, where error bars are 95% confidence intervals. (D)The mean and standard deviation of flies with quantifiable bacterial growth is shown in black with associated standard deviation (12.83 ($\pm$ 3.38) CFUs/ml on the log_2 scale).

Table 4.3: Model summaries for the effects of quantifiable presence of bacterial growth in infected flies (0 no presence, 1 for presence). (A) Model summaries of model analysing total sips is a negative binomial model, and (B) model analysing preference index, where 1 indicates preference for the 1:4 P:C food, 0 for no preference and -1 for preference for the 0:1 P:C food, is from a linear mixed effects model. Block was fitted as a random effect.

(A) Effects of quantifiable bacterial growth presence (1 for present, 0 for not) on total sips in infected individuals:						
	Estimate	Standard error	T value	Df	Chisq	Pr (>Chisq)
Intercept	5.21	0.48	10.79			
Bacterial growth (1)	-0.01	0.44	-0.03	1	0.0007	0.98
(B) Effects of quantifiable bacterial growth presence (1 for present, 0 for not) on preference index in infected individuals:						
	Estimate	Standard error	T value	Df	Chisq	Pr (>Chisq)
Intercept	0.06	0.22	0.27			
Bacterial growth (1)	-0.15	0.23	-0.65	1	0.42	0.52

4.5 Discussion:

A common sickness behaviour is reducing food intake post-infection (reviewed in Kyriazakis et al., 1998; Hite et al., 2020). When given a choice in food, infected individuals often decrease their overall eating and show a preference for diets that are beneficial for survival (reviewed in Hite et al., 2020). In a previous experiment using the same host-pathogen pair, although all individuals had higher survival and reproduction on higher P:C diets, the difference in survival between low and higher P:C diets was much greater with infection (Chapter 2). Therefore, we predicted that all flies would prefer the higher P:C diet, and this preference would be more pronounced in the infected flies. Regardless of infection treatment, there was no difference in the total amount of food consumed and no evidence of greater overall preference for the higher protein food when flies were given a short-term food choice test. While there was no overall preference in terms of preference index, individuals which ate above a certain total amount of sips ate more of the 1:4 P:C diet (20% protein). Infection or injury had no effect on this preference for the 1:4 P:C diet with total sips. Altogether, this suggests that flies prefer the 1:4 P:C diet but only above a certain number of sips in total, and infection does not change preference, regardless of potential survival benefit associated with consuming more of the food with a higher protein content.

Independent of infection, we predicted that all individuals would prefer the 1:4 P:C diet, as in a previous experiment uninfected and infected *D. melanogaster* had higher survival and reproduction on intermediate P:C diets (Chapter 2). Due to flies not eating a higher P:C ratio than 1:4, we were unable to measure diet preference between two diets with varying protein contents. Overall, we found that individuals above a threshold level of 150 total sips had a preference for the 1:4 P:C diet and below this threshold level of total sips had a preference of the 0:1 P:C diet. Previous macronutrient intakes studies in *D. melanogaster* report that flies have an similar intake ratio around 1:4 P:C, where individuals were given choices between two liquid diets in capillaries and food choice is measured for a longer time over several days (Lee et al., 2008; Jensen et al., 2015; Ponton et al., 2020). As

liquid diets are different to more standard laboratory feeding on solid diets, our experiment confirms *D. melanogaster* in the short-term will also choose this ratio when given solid diets, however individual variation in total feeding can affect this choice. While flies had a preference for the 1:4 P:C diet with higher total number of sips, this resulted in no overall food choice, suggesting a disconnect between overall preference and preference given the total number of sips. It is unclear whether previous studies have also seen similar variation between the two measures, as previous flyPAD studies only present data either for total sips taken or preference index separately (Itskov et al., 2014; Piper et al., 2017; Carvalho-Santos & Ribeiro, 2018; Steck et al., 2018). Our study highlights the need for consideration of individual variation in overall feeding. This may be especially important in future studies measuring food choice with infection, as infection can affect both total feeding and choice of diet (Ponton et al., 2011b, 2013). Measuring food intake over a few days or across days would aid in understanding whether this short-term food choice test is indicative of real preference after acclimating to the testing arena, and whether a longer time is required to capture changes in food choice.

It is unclear why total feeding affected food preference. One consideration is that total feeding as measured here may also include contact with food, and is not necessarily equivalent to total feeding. However, when sip data has been compared to manual videos of feeding, these two measures had high correlation (Itskov et al., 2014). As the experiment required a 21 hour long starvation period to motivate feeding, a factor that may have affected individual variation on total sips taken may be due to variation in individual starvation resistance (reviewed in Rion & Kawecki, 2007). Starvation may have altered how the flies adjusted to the experimental set up, and as only one hour of feeding was measured, it is unclear how this short time frame may have influenced feeding behaviour between flies. Therefore, to determine whether starvation affected the results, this experiment should be repeated with additional food choice protocols not requiring a starvation period (e.g. Deshpande et al., 2014; Marx, 2015) or different starvation period should be tested. The diet pair itself could have changed relationships between

preference index and changes in preference at different total intakes. We only used a single dietary pair and dietary preference can vary depending on the exact pair of diets offered (Maklakov et al. 2008; Fanson et al. 2009; Harrison et al. 2014, but see Jensen et al. 2015). Therefore, additional tests using multiple diet pairs will help to determine whether the diet pair itself affected the relationship between total sips and number of sips of each food. Using multiple diet pairs with long-term sampling would also help to understand changes in intake targets rather than short-term changes in diet choice. Finally, previous studies using the flyPAD have used much higher protein content diets as used here, up to 10% yeast (Itskov et al., 2014; Carvalho-Santos & Ribeiro, 2018; Steck et al., 2018), and further studies are required to understand why such variation occurs.

We found no evidence of food choice changing with infection, despite similar infection conditions having been shown to affect survival of infected flies on different P:C diets (Chapter 2). There are several potential explanations for these results. First, a study in *Spodoptera littoralis* caterpillars found that immune challenge with *Micrococcus lysodeikticus* lyophilised cells did not alter the intake target, however the resulting immune response measures such as lysozyme levels changed around the intake target with infection in comparison to the uninfected individuals (Cotter et al., 2011). It was proposed that instead of changing their food choice, infected individuals were changing their internal allocation of resources to alter various immune responses, or alternatively that this effect was due to not using a live infection (Cotter et al., 2011). Therefore, measurements of various immune responses in our host-pathogen system would allow inferences as to whether they show similar P:C related changes post-infection. Second, the lack of food choice with infection could be pathogen mediated (see Rao et al. (2017)), as the host increasing its P:C intake here would lead to higher survival post-infection, and therefore lower pathogen fitness. In contrast, dietary P:C had no effect on *P. entomophila* post-infection survival in *Blatta orientalis* cockroaches, however infected individuals chose a higher P:C ratio in comparison to uninfected individuals (Sieksmeyer et al., 2021), suggesting *P. entomophila* infection can lead to changes

in food choice without associated changes in survival, requiring further testing in other hosts. This pattern was suggested to be due a potential decoupling of immune response and dietary intakes in *B. orientalis* due to adaptations to processes such as detoxification (Sieksmeyer et al., 2021). Third, changes in diet choice can be pathogen specific, as individuals do not alter food choice with different pathogens (Ayres & Schneider, 2009; Shikano & Cory, 2016). Therefore, understanding these relationships and dynamics could help in determining why there was no change in food choice in this and other systems.

One additional consideration in lack of changes in food choice post-infection is the length of starvation post-infection treatments prior to food choice tests. Our aim was to assess food choice in similar infection conditions to a previous experiment, where individuals were given food prior to infection treatments (Chapter 2). From pilot testing, a long 21 hour starvation period post-infection treatments was required to motivate individuals to eat during the food choice assay (compared to no starvation in other studies (Itskov et al., 2014)). Changes in food intake post-infection may only be apparent close to post-infection (Kazlauskas et al., 2016). However, there is considerable evidence of altered food choices outside of this immediate time period post-infection, for example, from studies measuring food choice a few days post infection (Lee et al., 2006; Mason et al., 2014; Sieksmeyer et al., 2021). For studies measuring cumulative food intake across multiple days, individuals still decrease their food intake and changed their macronutrient intake (Povey et al. 2014; Dinh et al. 2019, but see Povey et al. 2009; Shikano and Cory 2016). We saw no changes in any food choice measure, suggesting that with *P. entomophila* infection in *D. melanogaster*, feeding closer to post-infection is important, or that there is no food choice change at all in this host-pathogen pair, as found with some pathogens (Ayres & Schneider, 2009; Shikano & Cory, 2016). To determine whether we potentially missed any changes in food choice, further experiments such as using a time course, measuring feeding continuously from infection for a few days and reversing the starvation and infection steps should be completed.

The infection method may have contributed to the lack of change in food choice. Infection dose can alter various host-pathogen interactions (Howick & Lazzaro, 2017; Wilson et al., 2020). In contrast, we found no difference in food choice or total sips taken in individuals with or without quantifiable bacterial load, suggesting that variation in infection load (as measured here) had no effect on food choice. We used a low estimated diluted dose, to test the flies in similar conditions as in a previous experiment where post-infection survival was affected by diet (Chapter 2). Perhaps as a result of a detection threshold, many flies in our experiment had no quantifiable bacterial load. Therefore, using a higher dose and using serial dilutions of doses would allow a test of whether food choice is affected by bacterial load at higher doses than used here. In addition, using a lower starting volume of LB broth in the CFU protocol (see Chapter 1, section 1.11.4) would allow for testing whether flies had CFUs which were missed in too high of an initial dilution strength. In *P. entomophila* systemically infected *Blatta orientalis,* only a higher dose corresponded to statistically significant changes in P:C intakes in comparison to wounding (Sieksmeyer et al., 2021). *P. entomophila* infection blocks food uptake in *D. melanogaster* larvae with an oral infection method (Vodovar et al., 2005; Liehl et al., 2006). The septic infection method used here likely bypassed the natural pathway of infection, and therefore potentially also the blockage of food uptake. Therefore, repeating this experiment with a more natural infection process and with higher dilutions of bacterial solution should help in determining whether infection alters food choice in this host-pathogen system. In addition, using serial dilutions of the bacterial broth would allow to estimate the effect of dose on food choice changes and what this potential detection threshold is.

4.6 Conclusion:

Overall, we found that *P. entomophila* infection had no effect on *D. melanogaster* short-term food choice. All treatment groups took the same number of sips in total and had a preference for the diet containing a protein source, which may have a survival and reproduction benefit for all individuals. This preference was only present when flies had taken above a threshold number of sips in total, and flies with lower intakes had a preference of the diet only including carbohydrates. Our results suggest that with *P. entomophila* infection, although *D. melanogaster* individuals may have a survival benefit post-infection on the higher P:C diet, they do not have a higher preference for the higher P:C diet post-infection in comparison to the injury and control uninfected individuals, or that the experimental design failed to detect these differences. We also saw no evidence of reduced food intake in infected flies, which is a commonly observed sickness behaviour. Altogether, our results suggest that *P. entomophila* infection does not alter host food choice in *D. melanogaster*, even though there is a potential survival benefit of eating more of the diet with a protein source, and therefore that food choice post-infection depends on the specific host-pathogen pair used. Further testing is required to understand whether this lack of food choice remains with higher infection doses, different techniques to measure food choice, including measuring intake targets, without a long starvation period or with other diet pairs.

Chapter 5:

Higher dietary protein:carbohydrate may improve *Drosophila melanogaster* survival by promoting tolerance but not clearance of *Pseudomonas entomophila* bacterial infection

5.1 Abstract:

Various forms of diet manipulation affect survival outcomes post-infection. In particular, changing dietary protein to carbohydrate (P:C) ratio influences survival, where diets with P:C ratios that increase survival post-infection are often associated with an increase in the expression of immune genes, reduced pathogen growth, and consequently, increased survival. In contrast, other studies have shown increased survival with no change in pathogen growth. In a previous study, female fruit flies (*Drosophila melanogaster)* infected with the bacterial pathogen *Pseudomonas entomophila* survived better on intermediate P:C, with very low survival on low P:C diets. To determine whether the increased survival on higher P:C was driven by individuals clearing the infection faster (increased resistance), or due to individuals tolerating the pathology of a given microbe load better (increased tolerance), here we measured survival, bacterial load and AMP gene expression of infected flies on two P:C diets at three time points post-infection. We found that although survival increased on the higher P:C diet, bacterial loads and AMP gene expression were similar across the two diets. As we found no general evidence for increased resistance with *P. entomophila* infection, our data suggest that increased survival post-infection may be driven by increased disease tolerance on higher P:C. This study highlights the growing evidence of pathogen-specificity and the complexity involved in diet-mediated effects on immunity and survival post-infection.

5.2 Introduction:

There is considerable variation in infection outcomes depending on how components of the host, pathogen or the environment interact (e.g. Lazzaro & Little, 2009; Wolinska & King, 2009; Vale et al., 2011; Barribeau et al., 2014; Zouache et al., 2014). Not all individuals die when exposed to the same infection dose, and there is considerable between-individual variation in survival outcomes post-infection (e.g. Howick & Lazzaro, 2014; Duneau et al., 2017). This variance can be explained by pathogen growth and variation in how the host is able to control this (generally called resistance), and can also arise due to variation in how the host can prevent or repair the damage caused by pathogen growth and tolerating the pathology of a given microbe load better (disease tolerance) (Ayres & Schneider, 2012; Medzhitov et al., 2012; Howick & Lazzaro, 2014; Kutzer & Armitage, 2016a). There is increasing evidence that diet can alter the outcome of infection, and the field of nutritional immunology focuses on how these outcomes change with modifications in diet (reviewed in Ponton et al., 2011b, 2013). Dietary effects on infection outcomes may depend on the effects of nutrients on the host or pathogen, or due to their interactions (reviewed in Ponton et al., 2011b, 2013). For example, a meta-analysis found that similar types of diet manipulations showed contrasting host mortality or survival responses with different pathogens across taxa (Pike et al., 2019). In a previous experiment in *Drosophila melanogaster*, individuals infected with *Pseudomonas entomophila* bacterial infection had altered post-infection survival of flies on different diets (Chapter 2). Here we measured how pathogen numbers and a component of the host immune response against bacteria change with time post-infection on two different diets.

Diet composition can have profound effects on an individuals' response to infection, including the host's resistance or disease tolerance (Ponton et al., 2013; Kutzer & Armitage, 2016a; Lissner & Schneider, 2018; Martins et al., 2019). Given that the host and pathogen share the same nutritional environment, host nutrition may affect pathogen growth directly by modifying the nutrients available for pathogen replication (Ponton et al., 2013; Cressler et al., 2014). Host nutrition may

also directly inhibit or slow down growth of the pathogen, for example, in *Spodoptera littoralis* caterpillars, higher dietary protein diet increases haemolymph osmolality, the concentration of solutes in the insect haemolymph, and this slows down growth of *Xenorhabdus nematophila* bacteria (Wilson et al., 2020). As diet can affect both pathogen growth through host resistance and bacterial growth conditions, combining measures of both pathogen load and host defence should aid in understanding how diet affects this variation with bacterial infection.

Insects are frequently the focus in studies investigating diet effects on the immune response (reviewed in Ponton et al., 2011b, 2013). One reason for this is that insects and vertebrate systems have many conserved pathways and similarities in organs involved in immunity and metabolism (reviewed in Ponton et al., 2011b, 2013; Galenza & Foley, 2019). One very general way to quantify immune response in insects with changing diet is to measure the overall lysozyme-like antibacterial activity (Cotter et al., 2011, 2019; Graham et al., 2014; Duffield et al., 2020) or haemolymph antimicrobial activity (Povey et al., 2009, 2014; Sieksmeyer et al., 2021). Greater resolution of antimicrobial activity can be achieved by measuring antimicrobial peptide (AMP) gene expression to understand how diet alters gene expression of specific AMPs. AMPs are produced either in the fat body and circulated in haemolymph, or locally, for example in the gut and trachea (reviewed in Lemaitre & Hoffmann, 2007; Hanson & Lemaitre, 2020). For bacterial infections, the type of peptidoglycan (PGN) associated with bacteria activates either the Toll or Imd signalling cascades, which both induce the production of different AMPs (reviewed in Lemaitre & Hoffmann, 2007). The Toll signalling cascade is activated by Lys-type PGNs, associated with mainly gram-positive bacteria, whereas the Imd signalling cascade is activated by DAP-type PGNs, associated with mainly gram-negative bacteria (reviewed in Lemaitre & Hoffmann, 2007). Some AMP genes such as Drosomycin can be expressed by both signalling cascades (De Gregorio et al., 2002). AMPs are under tight immune regulation and exhibit high levels of specificity for different types of bacterial pathogens (Hanson et al., 2019a).

Various methods of diet manipulation affect post-infection survival (Ponton et al., 2011b, 2013; Pike et al., 2019). One common diet manipulation is through dietary restriction (DR), where either the overall or a specific nutrient intake is reduced without malnutrition (reviewed in Mair & Dillin, 2008). In addition to the effects of DR on infection response, DR increases lifespan and reduces reproduction across a range of taxa (reviewed in Mair & Dillin, 2008). It is unsurprising that DR also affects survival post-infection, as DR affects several measures of the immune response in the absence of infection (e.g. Lee et al., 2006; Povey et al., 2009; Cotter et al., 2011). Reduced calorie intakes lead to lower survival post-infection (reviewed in Ponton et al. 2011, 2013, but see Ayres and Schneider 2009). However, such studies do not consider how the limitation of specific macronutrients may be causing this response (reviewed in Ponton et al., 2011b, 2013). Through experiments manipulating specific macronutrient intake or protein intakes, protein to carbohydrate (P:C) ratio has been identified as key in the infection response (reviewed in Ponton et al., 2011b, 2013), with a general pattern that infected individuals are more likely to survive on higher P:C post-infection (e.g. Peck et al., 1992; Lee et al., 2006; Povey et al., 2009; Cotter et al., 2019). However, higher survival post-infection has also been reported on lower P:C diets (Lee et al., 2017; Dinh et al., 2019; Ponton et al., 2020). This suggests that nutrition affects survival post-infection in a pathogen-specific way.

A common finding in studies focusing on diet effects on survival post-infection is that dietary P:C increases resistance, where bacterial loads are often lower on P:C diets with higher post-infection survival (Howick & Lazzaro, 2014; Lee et al., 2017; Dinh et al., 2019; Wilson et al., 2020). However, this decrease in bacterial growth is not present with all pathogens, suggesting instead higher tolerance on the diet associated with increased post-infection survival independent of bacterial clearance (Ayres & Schneider, 2009; Miller & Cotter, 2018). *Nicrophorus vespilloides* burying beetles infected with *Photorhabdus luminescens* were more likely to survive on lower protein to fat diets, but there was no associated change in bacterial load (Miller & Cotter, 2018). The effect of diet on the

host's ability to control bacterial loads may also be pathogen-specific. In *D. melanogaster,* caloric restriction led to increased survival despite no change in *Salmonella typhimurium* growth, while infection with *Listeria monocytogenes* resulted in both increased bacterial load and mortality in calorie restricted compared to control flies (Ayres & Schneider, 2009).

Many studies measure how diet changes host immune measures, with or without a live infection, which may explain changes in resistance with infection. Diet effects on host antimicrobial responses appear mixed. Depending on the study, higher P:C can increase lysozyme-like antibacterial (Cotter et al., 2011, 2019; Graham et al., 2014) or antimicrobial activity (Povey et al., 2014), antimicrobial activity can be higher on intermediate P:C (Povey et al., 2014), or diet can have no effect on antimicrobial activity (Duffield et al., 2020; Sieksmeyer et al., 2021). Diet can also affect AMP gene expression (Lee et al., 2017; Cotter et al., 2019; Ponton et al., 2020). For example, infected *D. melanogaster* on lower P:C had higher survival post-infection and higher AMP gene expression of many AMP genes on lower P:C (Lee et al., 2017; Ponton et al., 2020). *S. littoralis* caterpillars infected with *X. nematophila* bacteria had higher survival on higher, not lower, P:C diets and had the highest immune gene expression at intermediate P:C, however bacterial loads were not measured (Cotter et al., 2019). In this study, higher Moricin AMP gene expression was associated with a higher likelihood of dying post-infection (Cotter et al., 2019). When diets are altered in conjunction with AMP gene expression, bacterial loads are often not measured. However in one study, diets associated with higher survival, lower P:C diets, were also associated with lower bacterial load, suggesting higher gene expression of AMPs may have been involved in reducing bacterial loads (Lee et al., 2017). Taken together, these studies suggest that patterns between diet, survival and AMP gene expression may be complex and potentially pathogen-specific, and therefore the measurement of multiple AMPs is required to further understand how P:C affects specific AMPs.

In this study, we investigated the relationship between dietary P:C and both bacterial load and AMP gene expression in an attempt to improve our

understanding of diet-mediated effects on infection outcomes and whether diets associated with higher post-infection survival increase resistance (decreased pathogen load) or disease tolerance (no change in pathogen load) in a previously unmeasured host-pathogen pair. In a previous experiment, *D. melanogaster* infected with the bacterial pathogen *P. entomophila* had particularly poor survival on low P:C, with highest survival on intermediate P:C ratios (Chapter 2). *P. entomophila* is an entomopathogenic gram-negative bacteria, which was isolated from a wild *D. melanogaster* (reviewed in Dieppois et al., 2015). Infection with *P. entomophila* triggers the IMD signalling cascade leading to the downstream upregulation of several Imd-regulated AMP genes both systemically, and AMP genes such as Diptericin, Drosocin, Attacin C, Cecropin A1 and Drosomycin are produced locally in the gut with gut infection (Vodovar et al., 2005). As a strategy to overcome host immune responses including AMP gene translation to functional proteins, *P. entomophila* inhibits gut global translation by approximately 50% (Chakrabarti et al., 2012). This global gut translation inhibition also inhibits gut epithelium renewal, and this has been proposed to lead to gut tissue damage and subsequent host death (Chakrabarti et al., 2012). Even with this global translational blockage, induction of Diptericin and Attacin A AMPs in IMD mutant flies have increased resistance to *P. entomophila* infection (Liehl et al., 2006), however the AMP gene expression results should be interpreted with caution due to this inhibition (see also Cotter et al. (2019)).

To understand how a component of the host antimicrobial response and the bacterial load within a fly changes with dietary P:C with *P. entomophila* infection, we measured bacterial growth and AMP gene expression at three time points post-infection with female flies housed on two P:C diets (by manipulating yeast to sugar ratios). We expected two possible scenarios to explain the higher survival with higher P:C. If higher P:C increases resistance by improving the clearance of bacteria post-infection, then flies housed on higher P:C should present lower bacterial loads and higher AMP gene expression. Alternatively, if higher P:C increases disease tolerance, then we would expect no change in bacterial loads and subsequently on

AMP gene expression between flies housed on either diet, despite higher survival as previously observed (Chapter 2).

5.3 Methods:

5.3.1 Experimental flies:

Experimental flies were from eggs of the 71st generation of an outcrossed population derived from the *Drosophila melanogaster* genetic reference panel (DGRP) as described elsewhere (Chapter 1, section 1.11.1, and Appendix A). Flies were housed at 25°C with a 12:12 L:D light cycle, and were housed on modified Lewis food until the start of the experiment (Lewis 1960, see 1:6 P:C/14% protein diet in Table 1.1, Chapter 1, section 1.11.2). Adults used in the experiment were seven-day old age matched females, which were housed in mixed sex groups until infection treatments.

5.3.2 Infection treatments:

Seven day-old flies were either injured with a sterile pin prick ("injured control") or infected with *Pseudomonas entomophila* bacteria ("infected"), as described elsewhere (Chapter 1, section 1.11.3 and Chapter 3, Appendix C). As we wanted to collect flies for AMP and bacterial load assays at 16, 24, and 48 hours post-infection (see below), infection treatments were done in three separate blocks within a 4 hour time period on one day (48 hour time point infection treatments starting at 12:00, 24 hour time point at 14:00, and 16 hour time point at 16:00). To reduce variation in infection dose, the same bacterial solution was used for all blocks, which was kept at room temperature conditions. Prior to starting each block, the OD of the solution was quantified and diluted appropriately to correspond to an equivalent OD of 0.001 (Chapter 3, Appendix C). Post-infection, flies were individually housed on one of two diets (see below).

In total, more flies were infected than injured, due to lower expected survival post-infection (final sample sizes: injury = 274, infection = 380 flies). A similar number of flies were injured and infected in each infection time block (see Appendix E, Table S5.1 for full sample sizes per diet). From this total number, flies were either collected for AMP or CFU analysis at one of the three collection time points (in total 128 flies were collected for CFU analysis and 284 for AMP analysis),

or followed for survival analysis (sample sizes 31-89 per diet and infection treatment, see Appendix E, Table S5.2 for full breakdowns by infection time block and diet). Survival was monitored daily for five days (monitoring was stopped when no more deaths were observed).

5.3.3 Diets:

We used two diets: one diet with a low P:C ratio of 1:16 (corresponding to 5% protein content) and one intermediate P:C ratio of 1:1 (corresponding to 46% protein) using modified Lewis food (Lewis 1960, see Table 1.1, Chapter 1, section 1.11.2). These diets were chosen from a previous experiment in the same host-pathogen system (Chapter 2), which correspond to diets with either low or high survival post-infection.

5.3.4 Bacterial load (colony forming units, CFU/ml):

Bacterial load was measured at 16, 24 and 48-hours post-infection treatments as the number of colony forming units (CFUs) detected within individual flies, following Gupta et al. (2017, see Chapter 1, section 1.11.4). For each time point, 10 injured and infected flies per diet were individually tested and only flies alive at the point of sampling were collected. Flies were collected individually into Eppendorf tubes at the same time as the AMP gene expression collected flies (see below), however they were processed after the AMP gene expression protocol. We quantified the concentration of the inoculation culture by plating 5 µl of the diluted bacterial broth to estimate the number of viable bacteria per millilitre of bacterial broth (24.25 $\log_2$ CFU/mL). An additional 10 infected and injured flies from the last infection block were tested an hour post-infection treatments to estimate the initial infection dose. These plates showed no bacterial growth. This lack of growth suggests that the threshold for detection may be above the dosage used in this experiment and therefore, there may be a potential lower threshold for quantification of bacteria using this assay.

5.3.5 Antimicrobial peptide (AMP) gene expression:

At 16, 24 and 48-hours post-infection, 8 replicates of groups of 3 flies per infection and diet treatment were collected in Eppendorfs, homogenised in 80 µl of TRI Reagent Solution (Thermo Fisher Scientific), and stored at -70 °C until further processing. Only flies alive at the point of sampling were collected. RNA was extracted using Direct-zol RNA MiniPrep (Zymo Reseach) kit, and samples were processed in two sets. The final volume of each eluted RNA sample was 50 µl. Two samples were lost in the sample preparation process. All samples were checked for contamination using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific) directly after RNA extraction. All samples passed this check and were then stored at -80°C until further processing. Using 2 µl of each sample of extracted RNA, reverse transcription (RT) was completed using M-MLV RT (Promega) kit, with a final solution volume of 50 µl, using a 1:1 dilution in RNAse free water. Six non-RT controls were made with samples across the three time points, diets and treatment combinations, using 2 µl of each sample with same end dilution and volume as the samples undergoing the RT step. This full RT step process was completed twice through to have enough sample volume for qPCR. All cDNA samples and non-RT controls were stored in -20°C until qPCR.

We tested for differential gene expression for Cecropin A1, Attacin C, Diptericin, Drosocin, and Drosomycin AMP genes (see Appendix E, Table S5.3), as these have been shown to be upregulated during *P. entomophila* infection (Vodovar et al., 2005; Liehl et al., 2006). We used previously published AMP-specific primers (Bastos et al. 2017; Lee et al. 2017; Troha et al. 2018, see Appendix E, Table S5.3 for full primer details). All primers used in qPCR were tested for efficiency using a serial dilution (efficiencies ranged from 93-112%, see Appendix E, Table S5.34 for primer efficiency and temperature used for each primer in the annealing step). As a within-sample control and to normalise gene expression to a gene not involved in injury or infection process, we used a primer for a ribosomal protein Rpl32 (Appendix E, Table S5.3). The gene expression of Rpl32 has been shown to be on average relatively stable for flies housed on different P:C diets (Ponton et al., 2011a).

We used SYBR Green PCR Master Mix (Applied Biosystems) for qPCR, with a final reaction volume of 10 µl (1.5 µl of cDNA). On each plate, two technical replicates for each primer and sample were used to account for differences in pipetting. All plates included negative controls, with at least two non-RT samples, and two blanks (RNAse free water). Plates were ran on a StepOnePlus Real-Time PCR System (Applied Biosystems), using 40 cycles (with a 2 minute holding stage, cycling stage with 95°C for 10 seconds and annealing temperature for each primer for 30 seconds (Appendix E, Table S5.3), with cycles followed with a melt curve stage). Any samples with flags such as including error message reporting high standard deviations, multiple melting peaks or other issues were reran and were not included in the analysis with consistent errors (final sample sizes 6-8 replicates per diet, treatment and collection time point, see Appendix E, Table S5.4).

5.3.6 Statistical methods:

The data were analysed using R software, version 4.0.2 (R Core Team, 2014). All graphs were drawn using ggplot2 (Wickham, 2016). The R package survminer (Kassambara & Kosinski, 2018) was used to make Kaplan-Meier survival curves. Because the data did not conform to proportional hazards assumptions (global term of cox.zph function χ^2= 18,74, p = <0.001), we used an event history model following Moatt et al. (2019, Chapter 3). The event history models were implemented using a binomial GLMM using lme4 R package (Bates et al., 2015). Here, individuals were scored daily as 0 for alive and once as 1 for dead to calculate the per day mortality risk (here referred to as mortality). The model included the fixed effects of diet and infection treatment, and their interactions. We included random effects of day to account for differences in survival across days, and individual ID to account for multiple measures for an individual.

As many flies did not show quantifiable bacterial growth (38/60 flies with no bacterial growth, about 63%), we decided to analyse CFU data in two steps. First, we used Kruskal-Wallis tests to analyse if the number of CFUs/ml (log_2 transformed) differed between diets, either for all the time points together, or at each time point separately. CFU results of only flies with detectable bacterial loads

were analysed, however including all flies with or without detectable CFUs did not change the results (data not shown). Second, we asked whether the proportion of flies with quantifiable bacterial load changed between the diets with time post-infection. We analysed the absence or presence of bacterial growth across diets and time point post-infection using a binomial model (1 indicates bacterial growth was present and 0 indicates it was absent) using the lme4 R package. Diet, time post-infection and their interaction were added as fixed effects. Flies tested an hour post-infection had no bacterial growth present, and were not included in the analyses.

For the AMP gene expression qPCR analysis, we used the relative quantification method (Livak & Schmittgen, 2001). In short, the C_T values of the samples were normalised for the average C_T value of the housekeeping gene (Rpl32) run on the same plate (ΔC_T). For each diet and time point, the average ΔC_T for the injury group was used to normalise each sample of infected flies ($\Delta\Delta C_T$). To calculate the final fold change expression, the equation $2^{-\Delta\Delta Ct}$ was used and $\log_2$ transformed. For each AMP gene separately, $\log_2$ fold change was analysed with a linear mixed effects model using the lme4 R package, including diet, time post-infection, and their interaction as fixed effects. In each separate model, we added a random effect of plate number, to account for variation across plates. To analyse whether diet had an effect on AMP gene expression in the injury group and therefore potentially in the AMP gene expression analysis, the delta C_T values for injured groups were analysed with a similar model, however all AMP genes were included in one model. Therefore, we included diet, time post-infection, AMP gene, and their interactions as fixed effects. We added random effects of sample ID, to account for multiple measurements, and plate number, to account for variation across plates. To analyse whether there were any differential gene expression in the reference gene Rpl32, we ran a similar model on the average Rpl32 C_T values, without including AMP gene as a fixed effect. For displaying summaries of LRT results of main effects or two-way interactions, parameter estimates and associated standard deviations are from separate models not including the

associated two-, or three-way interactions. For the Attacin C AMP gene expression model, a post-hoc estimated marginal means test using the R package emmeans (Lenth, 2020) was completed to analyse which level of the interaction was significant.

5.4 Results:

Infected flies had higher mortality than flies in the injury control treatment (Figure 5.1A & 5.1B; Table 5.1; Infection = 2.39 (±0.56), χ^2= 37.23, p = <0.001). Regardless of infection treatment, higher dietary P:C reduced mortality (Figure 5.1A & 5.1B; Table 5.1; Diet χ^2= 28.25, p = <0.001, 1:1 P:C = -1.49 (±0.39), 1:1 P:C:Infection = -0.90 (±1.04), χ^2= 0.73, p = 0.39). This reduction in mortality with higher P:C appears greater in the infection group, however this was statistically not significant, most likely due to the change in mortality appearing in the same direction of reduced mortality in both infected and injured flies (Figure 5.1A & 5.1B; Table 5.1). Although the survival data do not conform to proportional hazards, qualitatively similar results were found with a Cox-proportional hazards model (Appendix E, Figure S5.1; Table S5.5).

Bacterial growth did not change over time, regardless of diet or time post-infection (Figure 1C & 1D; Kruskal-Wallis time post infection: χ^2= 2.54, p = 0.28; Diet overall χ^2= 0.85, p = 0.36; Diet analysed separately for each time point 16 hours χ^2= 2.25, p = 0.13; 24 hours χ^2 = 1.13, p = 0.29; 48 hours χ^2= 0.09, p = 0.77). Diet and time-post infection had no effect on the proportion of flies with quantifiable bacterial load, as both diet treatments had similar numbers of individuals with quantifiable bacterial load (Figure 1C & 1B; Table 5.2; 1:1 P:C = -0.81 (± 0.91), χ^2 = -0.89, p = 0.37; 24 hours = -1.25 (±0.94), χ^2 = -1.33, p = 0.18; 48 hours = -1.79 (±1.02), χ^2 = -1.76, p = 0.08).

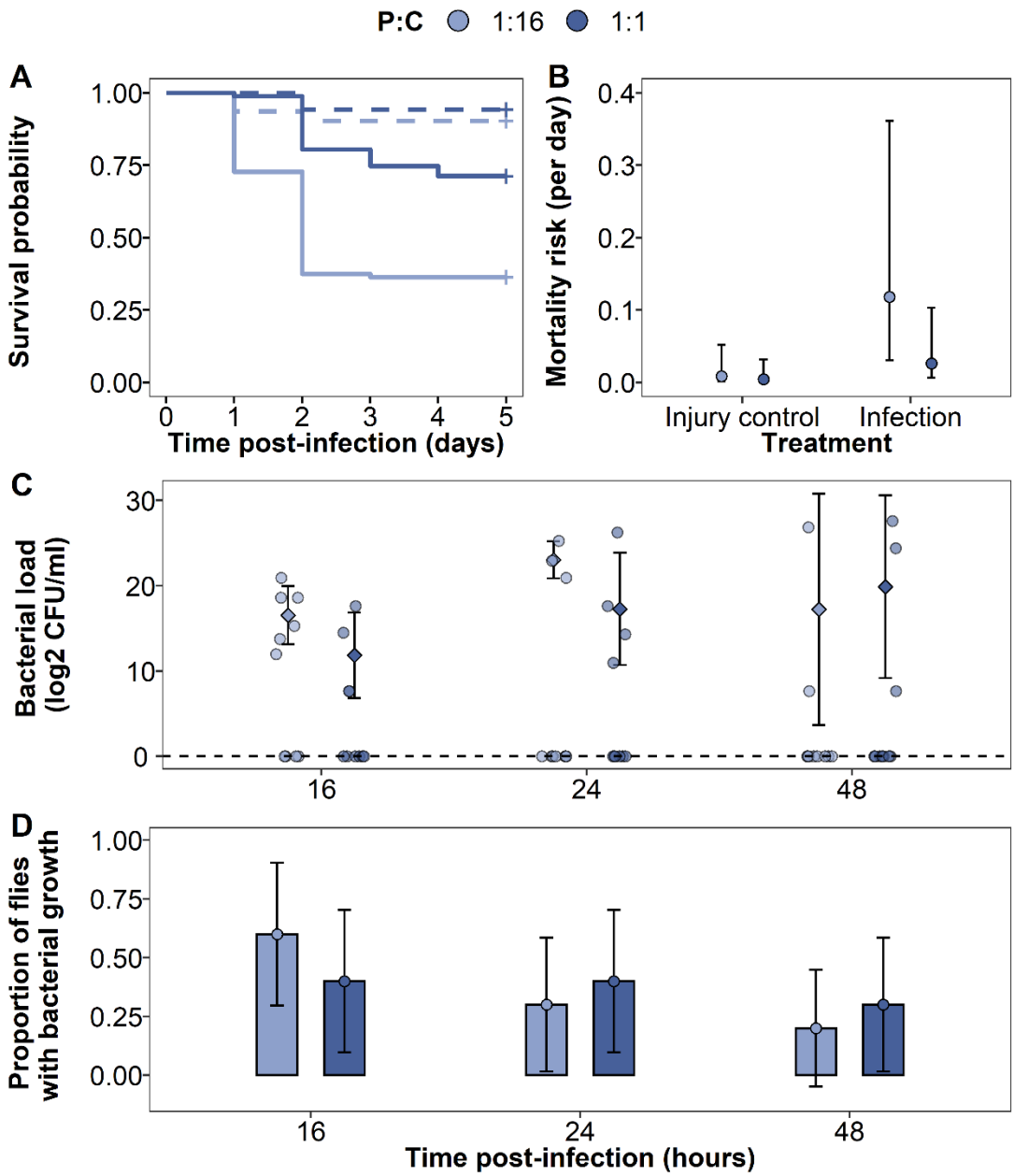

Figure 5.1: Effects of dietary P:C and time post-infection on (A, B) survival, and (C, D) bacterial load post-infection. Lighter purple lines show data for the lower P:C diet (1:16 P:C, 5% protein) and darker purple lines show data for the higher P:C diet (1:1 P:C, 46% protein). (A) Kaplan-Meier survival curves for injury control (dotted lines) and infected flies. Plus signs (+) indicate censored individuals at the end of the experiment. Sample sizes range from 31 to 89 individuals per diet and infection treatment (see Table S3). (B) Binomial event history model predictions for the effects of P:C on mortality risk (per day) post-infection treatment. (C) Bacterial load

shown as number of bacterial colonies present (log_2 CFU/ml) for each fly per diet and infection treatment (sample size 10 flies per diet and infection treatment). Dotted line shows y = 0. Diamond shapes show mean number of CFUs with associated standard deviations for flies that had quantifiable bacterial load. (D) Bacterial load shown as proportion of individuals with quantifiable bacterial load (each fly scored as 1 for having quantifiable bacterial load, 0 for no growth), and associated binomial model predictions. Error bars show 95% confidence intervals (C, D).

Table 5.1: A summary of a binomial event history model analysing effects of P:C and stress treatment on mortality risk (per day) post-infection treatment for (A) main effects parameter estimates and associated LRT test values and (B) for full model. Vial and Day are included as random effects. The significant results below significance level α = 0.05 are bolded. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	Z value	Chisq	Pr (> Chisq)
(A) Main effects parameter estimates and LRT test values:					
Intercept	-4.46	0.85	-5.23		
1:1 P:C	**-1.49**	**0.39**	**-3.81**	**28.25**	**<0.001**
Infection treatment	**2.39**	**0.56**	**4.29**	**37.23**	**<0.001**
(B) Full model parameter estimates and LRT test values for interactions:					
Intercept	-4.75	0.95	-5.03		
1:1 P:C	-0.71	0.96	-0.74		
Infection treatment	2.74	0.75	3.66		
1:1 P:C: Infection treatment	-0.90	1.04	-0.87	0.73	0.39

Table 5.2: Summary of a binomial model analysing effects of diet and time post-infection on the probability of a presence of bacterial growth (1 for presence, 0 for no presence).

	Estimate	Standard error	Z value	Pr (>Chisq)
Intercept	0.41	0.65	0.63	0.53
1:1 P:C	-0.81	0.91	-0.89	0.37
24 hours post-infection	-1.25	0.94	-1.33	0.18
48 hours post-infection	-1.79	1.02	-1.76	0.08
1:1 P:C:24 hours post-infection	1.25	1.31	0.95	0.34
1:1 P:C: 48 hours post-infection	1.35	1.39	0.97	0.33

The gene expression of AMPs in infected flies changed over time, differed according to P:C of the diet, and varied across AMP genes (Figure 5.2; Table 5.3). In general, compared to the reference of gene expression at 16 hours, there was a significant increase in AMP gene expression at 24 hours, and a significant reduction in AMP gene expression at 48 hours (Figure 5.2; Table 5.3; see associated estimates, standard errors and LRT test results in Tables S5.4-5.8). Diet had a significant effect on expression of Attacin C, Diptericin and Drosomycin AMP genes, however the direction of increased or decreased expression, and time-point where this difference was apparent, differed by AMP gene (Figure 5.2; Tables 5.3-5.8). Overall, although there were differential gene expression patterns with diet, the changes in fold change expression with diet are similar, and therefore suggest there are no biologically significant differences across diets (Figure 5.2). The only exception to this is the gene expression for Attacin C at 24 hours (Figure 5.2; Table 5.4). At 24 hours post-infection, the change in Attacin C gene expression on the higher P:C significantly decreased in comparison to the change in gene expression on the lower P:C (Figure 5.2; Table 5.4; 1:1 P:C = -1.53 ($\pm$0.80), χ^2= 18.47, p = <0.001; Diet:Time post-infection χ^2= 16.14, p =<0.001, 1:1 P:C:24 hours post-infection = -3.85 ($\pm$2.19)). Although there were some minor, but significant diet and time post-

injury effects on the injury control group (Appendix E, Figure S5.2; Tables S5.6-S5.8) or Rpl32 reference gene C_T values (Appendix E, Figure S5.3; Tables S5.9-S5.11), as the parameter estimates are overall very small, such differences should not change the overall main results of the gene expression analysis.

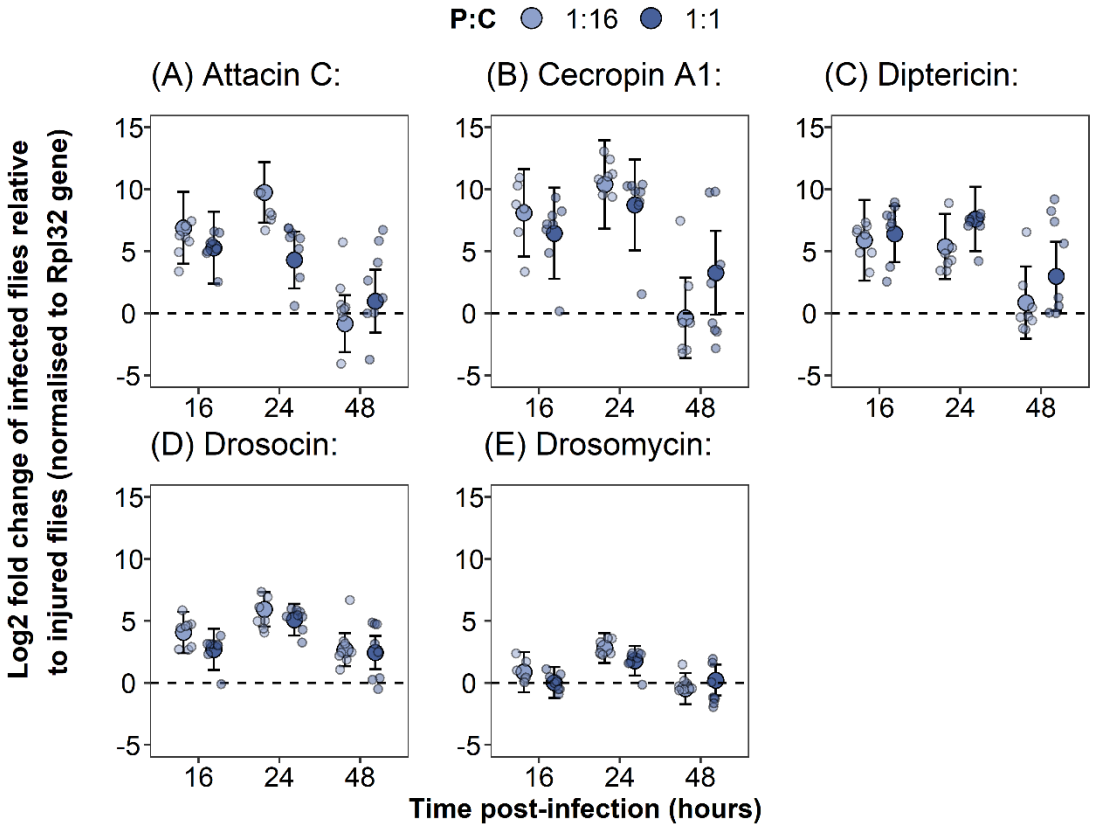

Figure 5.2: Effects of dietary P:C and time-post infection on AMP gene expression (log_2 fold change) of infected flies relative to injured flies (normalised to Rpl32 gene) for (A) Attacin C, (B) Cecropin A1, (C) Diptericin, (D) Drosocin and (E) Drosomycin AMP genes. Lighter purple points show data for the lower P:C diet (1:16 P:C, 5% protein) and darker purple points show data for the higher P:C diet (1:1 P:C, 46% protein). Data points correspond to 8 replicates of averages of groups of 3 flies per diet and time post-infection. Linear model predictions are shown as additional data points with associated error bars showing 95% confidence intervals. Dotted line shows y = 0.

Table 5.3: Summary of significant effects from separate linear mixed models for each AMP gene model analysing effects of diet and time post-infection on AMP gene expression. Upward arrows note significant positive model estimates (↑), and downwards signs note significant negative effects (↓) of the particular model term. Significance of main effects was tested using LRT tests from models not including the Diet:Time post-infection interaction and the significance of the interaction level was analysed with a post-hoc estimated marginal means test (see Table S10-S19 for model estimates and associated standard errors).

Model term	AMP gene				
	Attacin C	Cecropin A1	Diptericin	Drosocin	Drosomycin
1:1 P:C (46% protein)	↓		↑		↓
24 hours post-infection	↑	↑		↑	↑
48 hours post-infection	↓	↓	↓		
1:1 P:C: 24 hours post-infection	↓				
1:1 P:C: 48 hours post-infection					

Table 5.4: Summary of a linear mixed effects model of the effects of diet and time post-infection on log_2 fold change expression of Attacin C for infected flies compared to injured flies for (A) main effects parameter estimates and associated LRT test values and (B) full model. qPCR plate is included as a random effect. Significant results below significance level α = 0.05 are bolded. For the Diet:Time post-infection interaction, post-hoc estimated marginal means test for interaction: 24 hours post-infection, 1:16 P:C vs. 24 hours post-infection, 1:1 P:C = 5.46 (±1.13), t ratio = 4.84, p = <0.001; 48 hours post-infection, 1:16 P:C vs. 48 hours post-infection, 1:1 P:C = -1.81 (±1.15), t ratio = -1.57, p = 0.62. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	T value	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:					
Intercept	6.58	1.13	5.80		
1:1 P:C	**-1.53**	**0.80**	**-1.93**	**18.47**	**<0.001**
24 hours post-infection	**1.25**	**1.29**	**0.97**	**30.35**	**<0.001**
48 hours post-infection	**-5.39**	**1.34**	**-4.04**		
(B) Full model parameter estimates and LRT test values for interactions:					
Intercept	6.89	1.48	4.65		
1:1 P:C	-1.61	1.90	-0.85		
24 hours post-infection	2.86	1.66	1.73		
48 hours post-infection	-7.73	1.64	-4.72		
1:1 P:C:24 hours post-infection	**-3.85**	**2.19**	**-1.76**	**16.14**	**<0.001**
1:1 P:C:48 hours post-infection	**3.42**	**2.20**	**1.56**		

Table 5.5: Summary of a linear mixed effects model of the effects of diet and time post-infection on log_2 fold change expression of Cecropin A1 for infected flies compared to injured flies for (A) main effects parameter estimates and associated LRT test values and (B) full model. qPCR plate is included as a random effect. Significant results below significance level $\alpha = 0.05$ are bolded. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	T value	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:					
Intercept	7.39	1.11	6.65		
1:1 P:C	-0.43	1.06	-0.41	0.18	0.67
24 hours post-infection	**2.67**	**1.30**	**2.06**	**30.72**	**<0.001**
48 hours post-infection	**-5.92**	**1.30**	**-4.57**		
(B) Full model parameter estimates and LRT test values for interactions:					
Intercept	8.09	1.80	4.49		
1:1 P:C	-1.64	2.60	-0.63		
24 hours post-infection	2.29	2.53	0.91		
48 hours post-infection	-8.48	2.37	-3.58		
1:1 P:C:24 hours post-infection	-0.009	3.66	-0.002	4.96	0.08
1:1 P:C:48 hours post-infection	5.29	3.40	1.55		

Table 5.6: Summary of a linear mixed effects model of the effects of diet and time post-infection on log_2 fold change expression of Diptericin for infected flies compared to injured flies for (A) main effects parameter estimates and associated LRT test values and (B) full model. qPCR plate is included as a random effect. Significant results below significance level $\alpha = 0.05$ are bolded. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	T value	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:					
Intercept	5.04	1.00	5.06		
1:1 P:C	**1.90**	**0.85**	**2.23**	**6.86**	**0.009**
24 hours post-infection	**0.46**	**1.25**	**0.37**	**16.75**	**<0.001**
48 hours post-infection	**-3.89**	**1.19**	**-3.27**		
(B) Full model parameter estimates and LRT test values for interactions:					
Intercept	8.09	1.80	4.49		
1:1 P:C	-1.64	2.60	-0.63		
24 hours post-infection	2.29	2.53	0.91		
48 hours post-infection	-8.48	2.37	-3.58		
1:1 P:C:24 hours post-infection	-0.009	3.66	-0.002	2.32	0.31
1:1 P:C:48 hours post-infection	5.29	3.40	1.55		

Table 5.7: Summary of a linear mixed effects model of the effects of diet and time post-infection on log_2 fold change expression of Drosocin for infected flies compared to injured flies for (A) main effects parameter estimates and associated LRT test values and (B) full model. qPCR plate is included as a random effect. Significant results below significance level $\alpha = 0.05$ are bolded. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	T value	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:					
Intercept	3.73	0.56	6.71		
1:1 P:C	-0.68	0.47	-1.45	2.89	0.09
24 hours post-infection	**2.09**	**0.69**	**3.04**	**22.30**	**<0.001**
48 hours post-infection	**-0.77**	**0.69**	**-1.13**		
(B) Full model parameter estimates and LRT test values for interactions:					
Intercept	4.08	0.85	4.77		
1:1 P:C	-1.38	1.21	-1.14		
24 hours post-infection	1.85	1.11	1.67		
48 hours post-infection	-1.39	1.09	-1.28		
1:1 P:C:24 hours post-infection	0.54	1.47	0.37	1.34	0.51
1:1 P:C:48 hours post-infection	1.14	1.47	0.77		

Table 5.8: Summary of a linear mixed effects model of the effects of diet and time post-infection on log_2 fold change expression of Drosomycin for infected flies compared to injured flies for (A) main effects parameter estimates and associated LRT test values and (B) full model. qPCR plate is included as a random effect. Significant results below significance level $\alpha = 0.05$ are bolded. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	T value	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:					
Intercept	0.82	0.34	2.41		
1:1 P:C	-0.71	0.31	-2.33	6.99	0.008
24 hours post-infection	**1.88**	**0.44**	**4.29**	**20.19**	**<0.001**
48 hours post-infection	**-0.70**	**0.41**	**-1.71**		
(B) Full model parameter estimates and LRT test values for interactions:					
Intercept	0.87	0.83	1.04		
1:1 P:C	-0.84	1.05	-0.80		
24 hours post-infection	1.95	1.03	1.89		
48 hours post-infection	-1.33	1.05	-1.27		
1:1 P:C:24 hours post-infection	-0.19	1.14	-0.17	1.20	0.55
1:1 P:C:48 hours post-infection	1.53	1.35	1.13		

5.5 Discussion:

Changes in dietary P:C have been shown to affect survival outcomes post-infection, where individuals on diets associated with higher survival often show higher levels of resistance, such as lower bacterial loads and higher measures of various immune responses (e.g. Povey et al. 2014; Lee et al. 2017; Cotter et al. 2019; Ponton et al. 2020; Wilson et al. 2020). Dietary P:C can also alter disease tolerance, where higher survival on different P:C diets is observed despite not being associated with changes in overall pathogen loads (Kutzer & Armitage, 2016b; Miller & Cotter, 2018). The present work was motivated by the observation that *D. melanogaster* infected with *P. entomophila* had higher survival on higher P:C (Chapter 2). To understand whether this increased survival on higher P:C was due to increased resistance (faster clearing of bacteria), or increased tolerance (increased survival independent of changes in bacterial loads), we housed flies on one of two P:C diets and measured survival, bacterial loads and antimicrobial peptide (AMP) gene expression at three time points post-infection. AMP gene expression was measured to understand whether potential changes in bacterial load (resistance) were associated with changes in specific or all measured AMPs. The survival results were qualitatively similar to a previous study using the same host-pathogen system (Chapter 2), where survival post-infection was poor on the lower P:C diet post-infection. We found limited evidence of increased resistance on higher P:C, as bacterial loads and AMP gene expression were similar for individuals on the two diets, suggesting instead a role of increased tolerance on higher P:C with *P. entomophila* infection. As many individuals did not have quantifiable bacterial growth, these data should be interpreted with caution and further tests are required to understand whether diet has no effect on resistance with *P. entomophila* infection.

Quantifiable bacterial load or its presence was not affected by P:C, suggesting that increased survival on the higher P:C diet may not be associated with increased bacterial clearance. However, it should be noted that these results should be interpreted with caution, as there was a lack of data due to many flies

having no bacterial load ,and the variation in bacterial load of flies with quantifiable load. Disease tolerance is defined as the host's capacity to respond to or limit damage caused by the pathogen at a given pathogen load (reviewed in Ponton et al., 2013; Kutzer & Armitage, 2016a; Lissner & Schneider, 2018; Martins et al., 2019). Outside of P:C changes, different forms of diet manipulation can affect tolerance (Ayres & Schneider, 2009; Vale et al., 2011; Cornet et al., 2014; Kutzer & Armitage, 2016b; Knutie et al., 2017b; Miller & Cotter, 2018). For example, *N. vespilloides* burying beetles infected with *P. luminescens* had higher survival and phenoloxidase responses on lower protein to fat diets, but with no associated significant changes in bacterial load (non-significant trend towards higher on lower protein diets) (Miller & Cotter, 2018). In *D. melanogaster* infected with *Escherichia coli* or *Lactococcus lactis,* individuals had similar bacterial loads post-infection on diets which varied in both P:C and calorie content (Kutzer & Armitage, 2016b). With *E. coli* infection only, at 24 hours post-infection individuals on the lower P:C and calorie diet had higher fecundity tolerance (measured as percent change in adult offspring production of infected flies compared to wounded control at a given pathogen load) (Kutzer & Armitage, 2016b). This same lower P:C and calorie diet had the reverse effect on fecundity tolerance at 72 hours, suggesting tolerance measures outside of bacterial loads can change depending on measurement time and pathogen (Kutzer & Armitage, 2016b). This study varied both P:C and calorie content of diet, making conclusions based on P:C difficult, especially as a separate study using caloric restriction in infected *D. melanogaster* found increased tolerance, reduced resistance or no effect of diet on survival, depending on the pathogen (Ayres & Schneider, 2009). Here we show that when manipulating P:C, but keeping calories constant, P:C does not alter bacterial load as measured here, or the sample sizes were too low to quantify this effect.

As higher P:C suggest increased tolerance in this host-pathogen pair, future studies are required to understand possible diet-mediated tolerance mechanisms in this system. Tolerance mechanisms include limiting tissue damage resulting either from the pathogen directly, or from host immune responses (Medzhitov et

al., 2012; Ponton et al., 2013; Martins et al., 2019). In a study of two mice strains, one strain showed evidence of diet-mediated changes in intestinal permeability, where individuals on lower P:C diets infected with *Heligmosomoides polygyrus* had higher nematode loads and higher intestinal permeability at a given nematode load (Clough et al., 2016). With *P. entomophila* oral infection, *D. melanogaster* are hypothesised to die due to the accumulation of bacteria in the gut and associated damage to the gut epithelium (reviewed in Dieppois et al., 2015). We used systemic infection in this study and bypassed the natural infection route of *P. entomophila*. For DGRP lines infected systemically with *P. entomophila* and orally with *Erwinia carotovora carotovora* 15, susceptibility of lines did not correlate to a large degree, suggesting oral and systemic infection differences in susceptibility (Bou Sleiman et al., 2015). This study however did not use systemic infection with *P. entomophila*, making comparisons to our results challenging. Therefore, future studies using *P. entomophila* should incorporate oral infection with measures of the gut epithelium, for example measuring intestinal stem cell division (following Bou Sleiman et al., 2015), or other measures of gut pathology (following Regan et al., 2016). This would help to assess whether the patterns observed here with bacterial load or other unmeasured changes in tolerance such as gut permeability change with infection delivery with *P. entomophila* on different P:C diets.

One important point to note here is that many flies did not have quantifiable bacterial growth and therefore there is a possibility that the assay did not manage to capture bacterial load differences between the two diets. Although the bacterial solution used to infect these flies had a high number of viable bacteria per millilitre (about 24.25 $\log_2$ CFU/mL in the diluted bacterial solution), many flies tested an hour post-infection showed no quantifiable bacterial growth, suggesting that the initial infection dose was below a detection threshold of the assay and that this assay has a threshold for detecting bacterial growth in individual flies. We used a low estimated diluted dose to facilitate comparison with a previous study (Chapter 2) that used the same estimated diluted dose and detected (as here) considerable mortality costs of infection. We used individual flies to quantify

bacterial load to have a more accurate estimate of variation in bacterial load. Future studies should use groups of flies to analyse average changes in bacterial load similar to the AMP gene expression analysis here, repeat this experiment with higher infection doses, or use a smaller initial LB broth dilution to detect whether CFUs are present but at a lower quantity. Using a group of flies at this estimated diluted dose would allow to keep similar infection conditions that lead to large changes in mortality with diet, however would avoid the problem with a detection threshold.

Another consideration is that the changes in bacterial loads between the two diets may have been apparent earlier in the infection process and resolved at our first measurement time point of 16 hours. In *Tenebrio molitor* beetles, bacterial loads had significantly decreased 30 minutes post-systemic infection, where simultaneously antibacterial activity increased after 16 hours post-systemic infection (Haine et al., 2008). From our data, AMP gene expression at 16 hours post-infection was already upregulated in the infected flies, suggesting that bacterial loads may have reduced at this point. There is evidence that *P. entomophila* bacterial loads decline quickly post-infection, although these results are difficult to compare to our study as they used an oral infection method (Bou Sleiman et al., 2015). With oral infection of *P. entomophila*, resistant and susceptible DGRP fly lines had peaks of bacterial load between 0.5-1.5 hours post-infection, which reduced to near zero at 16 hours post-infection (Bou Sleiman et al., 2015). Similar time courses should be completed with systemic infection in *P. entomophila* to understand how quickly bacterial loads change post-infection with systemic infection. To test whether diet-mediated changes in bacterial load were present prior to our testing time points, further studies should include time courses closer to infection time.

Although there were several minor but significant changes in AMP gene expression, we found no clear biologically relevant differences between diets on the expression patterns of the measured AMP genes. Although Attacin C gene expression at 24 hours had a greater change in gene expression on the lower P:C

diet compared to the other AMP genes, we interpret this as noise. Previous studies focusing on changes in immune gene expression have found complex changes in such measures with diet (Cotter et al., 2019; Ponton et al., 2020). *S. littoralis* caterpillars with *X. nematophila* bacterial infections had higher immune gene expression of some of the measured immune genes at intermediate P:C diets, where peaks in gene expression were at slightly different protein intakes, when highest survival post infection was on the highest P:C diets (Cotter et al., 2019). Upregulation of the only AMP gene measured was also associated with quicker host death, suggesting increased AMP gene expression may sometimes be an indicator of disease severity (Cotter et al., 2019). In addition, functional assays highlighted that gene expression may not reflect the activity of the particular gene, especially when protein is limiting (Cotter et al., 2019). Further studies analysing diet effects in AMP gene expression and their activity are required to understand how measuring gene expression of AMPs reflect changes with diet in an infected individual. In *D. melanogaster* with *Micrococcus luteus* infection, increased survival and AMP gene expression was associated with lower, not higher, P:C diets, however 3 out of 9 genes showed no significant change with diet (Ponton et al., 2020). The results we present here, together with previous findings, suggest that changes in AMP gene expression with diet may vary depending on the diet composition, pathogen and the AMP measured.

To our knowledge, only one other study has measured P:C effects on both antimicrobial activity and bacterial loads (Lee et al., 2017). *D. melanogaster* infected with *Pseudomonas aeruginosa* on lower P:C diets had higher survival, lower bacterial loads and higher expression of Diptericin, Drosocin and Attacin A AMP genes (Lee et al., 2017). This study used diets that varied in both calories and macronutrients, and therefore the increase in AMPs on lower P:C may have been due to caloric restriction, the reduction in P:C, or the interaction between the two. For survival and associated bacterial loads, this study also included results with isocaloric P:C changes. Even though survival post-infection was again higher on the lower P:C diet, the bacterial loads were much more comparable on the two diets,

suggesting effects of tolerance with changes only in P:C (Lee et al., 2017). Here we show that with only changes in P:C, AMP gene expression patterns changed with diet slightly, but not substantially, suggesting that these differences are not biologically very important and not able to result in differences in bacterial growth. Studying how much of the genes that are expressed translate to functional activity (following Cotter et al. (2019) would aid in understanding whether low P:C diets differentially affect translation, further changing AMP gene functionality with infection. Further studies incorporating P:C at various caloric contents may aid in understanding if different tolerance and resistance mechanisms are affected differently when different components of diet are changed.

5.6 Conclusion:

Overall, with *P. entomophila* infection in *D. melanogaster*, higher dietary P:C increased survival post-infection, however there was no quantifiable change in bacterial load and no large scale changes in AMP gene expression with P:C. Our results indicate that P:C may affect tolerance mechanisms with *P. entomophila* systemic infection, adding evidence of the importance of diet-mediated changes in disease tolerance and pathogen-specificity. Overall, additional studies are required to understand these dynamics further, and to determine whether there was no change in bacterial load with a higher sample size of individuals with quantifiable bacterial load.

Chapter 6:

General discussion

The most consistent environmental manipulation to extend lifespan and delay ageing is dietary restriction, where the overall caloric or a particular nutrient intake is reduced, without malnutrition (reviewed in Mair & Dillin, 2008; Fontana et al., 2010). Due to its prevalence across a range of taxa, many studies have focused on DR, or DR mimetics, as a potential application in humans to aid with an ageing society (reviewed in Speakman & Mitchell, 2011; Le Couteur et al., 2016; Balasubramanian et al., 2017). However, DR responses have been found to vary due to many factors (e.g. Magwere et al., 2004; Liao et al., 2010; Speakman & Mitchell, 2011; Nakagawa et al., 2012). In addition, one of the more recent DR evolutionary hypotheses proposes that additional stressors of injury and infection in laboratory conditions should remove the lifespan benefit of DR (Adler & Bonduriansky, 2014).

This thesis aimed to improve our understanding of the evolutionary basis of DR and its generality across environments. To do this, I addressed the following questions: (i) Do additional stresses of injury and infection remove the lifespan benefit of DR, and are some diets better for *D. melanogaster* survival post-infection with *Pseudomonas entomophila?* (ii) Does larval dietary macronutrient manipulation affect adult life-history traits and survival post-infection? (iii) Do infected *D. melanogaster* individuals have altered diet preference post-infection with *P. entomophila*? and (iv) Does diet affect host resistance or disease tolerance with *P. entomophila* infection? In this chapter, I will first outline the main findings of each chapter. Then, I will discuss the implications of this research, including some limitations, and explain how future work can be completed to help answer remaining questions.

6.1 Key findings:

6.1.1 Chapter 2: DR responses with injury and infection treatments

In chapter 2, I used a combination of 10 P:C diets and applied injury and infection stress treatments to adult female *D. melanogaster*. First, I found that the typical DR response to decreasing P:C (up to a point), the increase in lifespan and decrease in ageing and reproduction (e.g. Lee et al., 2008; Skorupa et al., 2008;

Bruce et al., 2013), was unaffected by stress treatment. One measure of ageing, gut integrity, was not maintained at a higher level with DR during the lifetime of a fly, however as only a small number of flies developed the smurf phenotype (the measure of gut integrity used in this study), these patterns should be interpreted with caution. Taken together, the prediction of the nutrient recycling hypothesis (NRH), stating that the DR lifespan extension response should be removed with additional stressors, was not met (Adler & Bonduriansky, 2014). The hypothesis does not make a distinction between surviving infection in the short-term in comparison to later-life survival, and focuses on DR effects on overall survival (Adler & Bonduriansky, 2014). Future studies should focus on this distinction with a higher sample size and determination about whether flies were infected to test whether diet affects these two processes differently. Although lifespan and reproduction peaked at intermediate P:C, reproduction peaked at a slightly higher P:C ratio than lifespan, and therefore, these results were more consistent with the proposed diet-mediated trade-off between lifespan and reproduction, as proposed by the resource reallocation hypothesis (RRH, Holliday 1989; Shanley and Kirkwood 2000).

I also found that infection with *P. entomophila* affected several life-history trade-offs. Infection affected lifetime survival post-infection, where infected individuals had very low survival on low P:C, similar to previous survival results from other host-pathogen systems (e.g. Lee et al. 2006; Cotter et al. 2019; Wilson et al. 2020, but see e.g. Lee et al. 2017; Dinh et al. 2019; Ponton et al. 2020). There is a possibility that diet may affect survival close to infection and on later-life survival in a different way, however due to the small sample sizes of flies in each diet by stress treatment combinations, this could not be tested in a statistically robust way. When accounting for the shorter lifespan of infected flies, infection had no effect on lifetime reproduction. Finally, infection did not substantially affect ageing patterns.

6.1.2 Chapter 3: Larval diets with adult injury and infection treatments

In chapter 3, I reared larvae on five P:C diets and applied injury and infection treatments in adult flies 7 days post-eclosion. In contrast to previous studies suggesting that larval diet may affect adult infection responses, I found that larval P:C had no effect on adult survival post-infection (Fellous & Lazzaro, 2010; Kelly & Tawes, 2013). Larval P:C had no effect on adult lifespan, a trait where previously early-life diet has had contrasting effects (e.g. Houslay et al., 2015; English & Uller, 2016; Davies et al., 2018). Larval diet had no consistent effect on survival of flies close to the application of stress treatments or on later-life survival, however larger sample sizes are required to test whether these patterns are statistically robust. Similar to previous studies, I found that larval P:C affected lifetime and early-life reproduction, where intermediate larval P:C maximised reproduction (Matavelli et al. 2015; Rodrigues et al. 2015, but see Silva-Soares et al. 2017; Kim et al. 2019). Although infection reduced lifespan and reproduction, larval diet did not interact with these responses, other than small, but significant, effects on ageing in egg production. However, broadly the patterns of ageing in egg laying were similar across diets. Finally, similar to some previous studies, I found that intermediate P:C reduced development time to adulthood and that higher P:C slightly increased egg-to-pupae and egg-to-adult viability (reviewed in Nestel et al. 2016), but dietary P:C had no effect on pupae-to-adult viability.

6.1.3 Chapter 4: Food choice post-infection with *P. entomophila*

In chapter 4, I measured how *P. entomophila* infection affected short-term food choice in *D. melanogaster* when flies were given a choice between two P:C diets. I found that regardless of injury or infection treatments, there was a threshold number of total sips, after which individuals preferred the higher 1:4 P:C diet (20% protein) in comparison to a diet with no protein (0:1 P:C, 0% protein). This lack of change in food choice post-infection was in contrast to previous studies, where a common finding is that infected individuals decrease overall food intake and prefer diets associated with higher post-infection survival (reviewed in Ponton et al., 2013; Hite et al., 2020). These results provide further evidence of

host-pathogen pair specificity in food choice, as *P. entomophila* infection in another host species, *Blatta orientalis* cockroaches, reduced overall feeding, and infected individuals preferred a higher P:C diet, although survival was not affected by dietary P:C (Sieksmeyer et al., 2021). In addition, these results indicate that total feeding can affect food choice analyses, and therefore should be accounted for in future studies, as individuals only had a preference for the higher P:C diet after eating a specific number of sips in total.

6.1.4 Chapter 5: Diet effects on resistance or tolerance with *P. entomophila*

In chapter 5, I measured whether higher P:C increased resistance (reduced pathogen loads and higher immune response) or disease tolerance (individuals tolerate the pathology of a given pathogen load) (reviewed in Ponton et al., 2013; Kutzer & Armitage, 2016b; Lissner & Schneider, 2018). This was done by measuring bacterial load and gene expression of five antimicrobial peptide genes of *P. entomophila* infected *D. melanogaster* housed on two P:C diets. I found that although survival post-infection was again higher on higher P:C (as seen in Chapter 2), bacterial loads and AMP gene expression were the same across diets. Therefore, resistance may not have been affected by diet, but I found evidence of diet apparently increasing disease tolerance, as survival differed between the diets with no change in pathogen loads. However due to small sample sizes of flies with quantifiable bacterial loads, further testing is required to determine whether diet affected bacterial growth. Although in some host-pathogen pairs diet increases tolerance (Kutzer & Armitage, 2016a; Miller & Cotter, 2018), many previous host-pathogen pairs report higher resistance, for example where individuals on P:C diets associated with higher survival have lower pathogen loads (e.g. Lee et al., 2017; Dinh et al., 2019; Wilson et al., 2020). These results add to growing evidence of host-pathogen pair specificity in diet effects on infection outcomes, and complexity of diet effects on disease resistance and tolerance across systems.

6.2 Key implications:

6.2.1 Evolutionary theories of DR - resource recycling or reallocation?

6.2.1.1 No evidence for the Nutrient Recycling Hypothesis (NRH):

Results from Chapter 2 indicate that DR responses remain with infection and injury, and therefore provide the first evidence against the prediction of the NRH that states additional stressors such as injury and infection should remove the lifespan benefit of DR (Adler & Bonduriansky, 2014). Several previous studies have tested other predictions from the NRH and generally find that these predictions are not met (Zajitschek et al., 2016; Mautz et al., 2019; Travers et al., 2020). For example, wild *Protopiophila litigata* antler flies had increased mortality with protein supplementation (Mautz et al., 2019), providing evidence against the NRH prediction that DR would not affect lifespan outside of benign laboratory conditions (Adler & Bonduriansky, 2014). This pattern had less support in one of the tested years (Mautz et al., 2019), and therefore more tests are required to confirm these findings. In addition, against the prediction that reproduction should decline under DR if autophagy is inhibited (Adler & Bonduriansky, 2014), this was not found in *C. elegans* (Travers et al., 2020). From the results from Chapter 2, together with previous findings (Zajitschek et al., 2016; Mautz et al., 2019; Travers et al., 2020), the NRH does not appear to explain the lifespan extension under DR.

Here, I used injury and infection stressors to test the predictions of the NRH, as they are proposed to be important stressors in wild conditions (e.g. Hoffman & Hercus, 2000; Adamo, 2020), and are named as testable stressors in the hypothesis itself (Adler & Bonduriansky, 2014). Another stressor that the NRH proposes may remove the lifespan benefit of DR is temperature (Adler & Bonduriansky, 2014). A study in *D. melanogaster* provides evidence that a range of temperatures (13-33 °C) may not remove the DR lifespan extension (Kim et al., 2020). Increasing temperature reduced male and female lifespan and reduced the decrease in lifespan with higher P:C (Kim et al., 2020), although there still appeared to be a slight decrease in female lifespan on the highest protein diets and male lifespan

appeared to peak at the lowest P:C even at higher temperatures. Female egg production peaked at 1:4 P:C, however at lower temperatures, egg production rate did not change with P:C (Kim et al., 2020). It should be noted that this study did not report how the curvature in lifespan or reproduction with P:C changed with temperature, but it was stated that at higher temperatures lifespan became less sensitive to changes in diet (Kim et al., 2020). Therefore, it cannot be stated whether at the extreme temperatures, the DR responses were still statistically significant, and further studies or analyses are required to confirm these findings (Kim et al., 2020). This study used a wild type *D. melanogaster* strain and solid chemically defined diets (Kim et al., 2020). In this thesis I used an outbred population of *D. melanogaster* and manipulated dietary yeast:sugar ratios, and therefore show that these responses with stressors are be present outside of tests on a single genotype and on diets which are commonly used in laboratory tests (e.g. Lee et al., 2008; Skorupa et al., 2008; Bruce et al., 2013).

From the results in this thesis, together with previous findings, it appears that DR responses are still present with additional stressors, however they may not appear at the extreme levels of temperatures. Overall, current evidence indicates that the NRH does not explain the lifespan extension with DR (Zajitschek et al., 2016; Mautz et al., 2019; Travers et al., 2020). It should be noted that, to the best of my knowledge, not all of the predictions from the NRH hypothesis have been tested (Adler & Bonduriansky, 2014). In addition, further studies in the wild are required to state whether DR extends lifespan in wild conditions and in different species (but see Mautz et al., 2019). These further tests of predictions are required to understand whether components of the hypothesis are valid, although current evidence indicates the NRH does not explain the lifespan extension of DR (see also discussion in Moatt et al. 2020; Regan et al. 2020).

Tests of how diet affects life-history traits have only included a single stressor. Outside of testing whether the NRH explains lifespan extension of DR, further studies combining stressors such as variation in diet, temperature and infection and injury status, should be completed to understand how multiple

environmental variables affect DR responses under even more stressful conditions. This would aid in our understanding about whether the certain combinations or high levels of stressors remove the lifespan benefit of DR, although here I have shown that infection combined with a septic infection method that includes injury, does not remove the lifespan benefit of DR even though mortality post-infection was high. Furthermore, as proposed by a shift in perspective of the current evolutionary and mechanistic processes underpinning DR and life-history trade-offs as a plastic response framework, an additional future avenue of research should be to understand effects of combinations of relevant environmental cues on life-history traits (Regan et al., 2020). Here, diet is only considered as one cue, which can be combined with other predictive cues of the environmental, such as photoperiod, water levels, and humidity, to understand, for example, how potential environmental predictive cue mismatches affect life-history traits or how multiple environmental cues affect plastic responses (Regan et al., 2020). This would also help in determining the relevance of diet manipulation in more ecologically relevant contexts compared to studies where only diet is manipulated (Regan et al., 2020).

6.2.1.2 Evidence for the Resource Reallocation Hypothesis (RRH)?

Similar to previous studies, both reproduction and lifespan peaked at intermediate P:C, where reproduction peaked at a slightly higher P:C ratio than lifespan (e.g. Skorupa et al. 2008; Lee 2015; Jang and Lee 2018; Kim et al. 2020, but see results for males in Jensen et al. 2015). As both traits did not peak at the same P:C ratio, this suggests there was a diet-mediated trade-off between lifespan and reproduction, therefore providing more evidence that the RRH explains the lifespan extension of DR (Holliday, 1989; Shanley & Kirkwood, 2000).

However, it has been suggested that the finding that reproduction and lifespan are not maximised on a single diet may not be due to an allocation trade-off between the two traits, and instead may be a constraint of the diet not allowing for both processes to be maximised (reviewed in Moatt et al., 2020). There is

increasing evidence that reproduction and lifespan do not always trade-off with each other (e.g. Speakman & Mitchell, 2011; Jensen et al., 2015; Piper, 2017). Consequently, further studies are required to understand whether the RRH explains the lifespan extension with DR, for example by radiolabelling protein in diets to track how much is allocated into somatic maintenance or reproduction (Moatt et al. 2020, following O'Brien et al. 2008). Radiolabelling protein in diet could also aid in understanding whether infection affects this allocation of protein into reproduction or lifespan, and therefore whether infection affects this trade-off (see also section 6.2.3).

To determine whether one of the other DR evolutionary hypotheses explain DR responses outside of the two this thesis focused on, predictions from other DR evolutionary hypotheses such as the toxic protein (Fanson et al., 2009, 2012, see discussion in Moatt et al. 2020), or clean cupboards hypotheses (Speakman, 2020) should be tested. Considerations from a more ecologically relevant shift in perspective, where diet is one of the cues an individual is responding to (see above and Regan et al., 2020), should be applied in future studies to help understand why DR extends lifespan (see also Moatt et al. 2020; Regan et al. 2020). As changes in micronutrients or specific or groups of amino acids can affect DR responses (Grandison et al., 2009; Le Couteur et al., 2020; Zanco et al., 2020), further studies should repeat the experiments here, but manipulate more detailed aspects of the diets such as particular amino acids or micronutrients. This would help to determine which aspect of diet or interaction between components of diet are driving changes in life-history traits seen here with broader changes in P:C. Overall, the results in this thesis are more in line with the RRH, however future studies are required to understand DR responses further.

6.2.2 Effects of dietary macronutrient manipulation in early- and later-life:

6.2.2.1 Effects of larval macronutrient manipulation on lifespan:

Understanding whether larval diet affects adult lifespan and ageing are paramount in determining how early-life condition alters lifetime traits, especially when studies often only focus on adult diet effects on these traits (e.g. Lee et al., 2008; Solon-Biet et al., 2014; Jensen et al., 2015). In chapter 3, similar to some previous findings, I found that larval diet did not affect lifespan when adult diet was kept constant (Tu & Tatar, 2003; Houslay et al., 2015; Davies et al., 2018). There was no consistent effect of larval diet on survival close to stress treatments, where only one model with a low sample size of individuals indicated that intermediate larval protein diet may increase later-life survival when individuals that died close to infection were removed from the analysis. Due to this inconsistency across models, further research with further sample sizes are required to test whether larval P:C has differential effects on surviving infection or later-life mortality.

Previous research into effects of larval diet manipulation on adult lifespan have had mixed results, where lifespan is maximised on low (Economos & Lints, 1984), intermediate (Runagall-McNaull et al., 2015; Kim et al., 2019), or high larval P:C (Duxbury & Chapman, 2020). In addition, a meta-analysis including invertebrate and vertebrate species found that overall early-life diet has no effect on adult lifespan, and suggested that early-life diet effects may be due to laboratory studies not including sufficiently high enough sources of mortality (English & Uller, 2016). In contrast to this suggestion, here I show that with additional stressors of injury and infection, and therefore potentially testing these patterns in a more biologically relevant environment compared to benign laboratory conditions, survival patterns due to larval dietary environment did not change. Therefore, these results provide evidence that with even with increased mortality from infection and a stressor of injury, larval P:C had no effect on lifespan.

It should be noted that I did not change larval and adult diets in a factorial way, and therefore there is a possibility that costs or benefits to adult lifespan may appear with certain combinations, as found in previous studies also changing larval diets in *D. melanogaster* (Davies et al., 2018; Duxbury & Chapman, 2020). With larval and adult diet mismatches, adult diet was still the main determinant of lifespan (Davies et al., 2018; Duxbury & Chapman, 2020), further suggesting minor effects on lifespan depending on the larval diet. Therefore, although effects of early-life diet on adult lifespan are mixed, the results here together with previous findings suggest that more probably early-life diet does not consistently affect lifespan, or that such consequences only appear in specific adult environmental conditions, which have not yet been fully understood.

One potential reason for the lack of changes in lifespan is that flies on the 1:6 P:C diet as adults were able to compensate for their poor dietary larval feeding (compensatory mechanisms reviewed in Raubenheimer and Simpson 1993). An additional suggestion is that the mortality patterns in adults may have adjusted to the current dietary environment regardless of larval feeding, and therefore mortality rates regardless of larval feeding were similar for all adults (see effects of changing diets in adults Mair et al. 2003, but see fitness costs of diet switching in McCracken et al. 2020a). Future studies should be completed to understand this variation in lifespan, including measuring food choice post-eclosion after changes in larval macronutrient manipulation and combinations of multiple matching and mismatching larval and adult P:C diets to measure changes in mortality patterns. Furthermore, previous studies have not applied nutritional geometry (GF, reviewed in Simpson and Raubenheimer 2012; Simpson et al. 2017) approaches to larvae to measure adult lifetime life-history traits. As I only manipulated P:C ratios without changing calories, applying GF including changes in calories to larvae would uncover whether and how different nutrients or their interactions affect adult life-history traits.

6.2.2.2 Considerations for future insect studies manipulating macronutrients:

Although here adult dietary environment had a greater effect on adult life-history traits than larval diets (Chapter 2 and 3), these results indicate that variation in larval diets should still be minimised when adult dietary macronutrients are manipulated. I found that increasing larval P:C up to a point increases early-life and lifetime reproduction, however effects on other traits such as ageing in egg laying were not as affected by larval P:C, as these small but significant effects were not as apparent across diets (Chapter 3). This increased reproduction when developing on higher P:C did not affect lifespan. Larval dietary environments should be controlled for to minimise the potential decrease in lifespan with increasing reproduction, as reproduction is generally considered to trade-off with lifespan (reviewed in reviewed in Stearns 1989, 2000; Shanley and Kirkwood 2000). As larval diet availability is affected by larval density, where more larvae leads to less food available to an individual (reviewed in Than et al., 2020), especially variation in larval density should be controlled as much as possible.

6.2.2.3 Which diets are more optimal for which traits?

These results also add to previous evidence that in insects, optimal P:C diets for different traits vary (e.g. Jensen et al., 2015; Rodrigues et al., 2015; Jang & Lee, 2018), and that in each developmental stage different life-history traits are prioritised (Lee et al., 2008; Boggs, 2009; Jensen et al., 2015). In general, intermediate P:C diets were optimal for most traits larval and adult traits, except for egg-to-pupae and egg-to-adult viability, which were slightly higher on higher P:C. Therefore, these results suggest that intermediate P:C diets are associated with higher fitness, however the exact P:C diets where this occurs differs between traits.

One previous GF study using solid diets with *D. melanogaster* also measured adult and larval diet manipulation separately and found similar results to ones presented here, except that egg-to-adult viability was highest at a lower P:C (Jang & Lee, 2018). However, the decrease in viability with P:C was not as steep at higher

concentrations of both nutrients (higher caloric values) (Jang & Lee, 2018), where the viability patterns became more comparable to the results in Chapter 3. This suggests these differences between studies may arise due to changes in calorie content. Effects of diet on viability can further vary by study, as three studies applying GF in *D. melanogaster* larvae found different patterns of diets on development to adulthood (Rodrigues et al., 2015; Gray et al., 2018; Jang & Lee, 2018). This suggests that further research into larval development mechanisms are required, for example to understand whether some amino acids are more important for growth in larvae and differ between studies (see effects of amino acids on larval development in Britton and Edgar 1998; Colombani et al. 2003; Chang 2004). In comparison to this earlier study (Jang & Lee, 2018), here I used an outbred population of *D. melanogaster* (similar to study only measuring larval traits Rodrigues et al., 2015). Therefore, the results from this thesis highlight that patterns seen in larval and adult macronutrient studies are present even when multiple genotypes are included, and adult environments are manipulated to include biologically relevant stressors outside of a benign laboratory condition.

6.2.3 Current dietary environment affects post-infection life-history traits:

6.2.3.1 Infection alters diet-mediated lifespan patterns:

While nutrition is an important trait that affects survival outcomes (reviewed in Ponton et al., 2011b, 2013), to date it has not been measured whether changing only the larval diet in insects affects this process and responding to injury in adulthood, and so whether an individual's early-life environment affects its ability to respond to key environmental stressors in adulthood. For holometabolous insects, where larvae are often confined to one diet source, larval diets may be important determinants of fitness (reviewed in Boggs, 2009), and consequently understanding how larval diet affects adult infection responses is important. Here, by using the same *P. entomophila* pathogen and measuring adult post-infection traits with adult (Chapter 2) and larval diets (Chapter 3), these results indicate that only the current adult environment affects survival outcomes post-infection, as

changes in larval diet had no effect on post-infection traits when adult diet was kept constant. I did not measure effects of larval diet on larval survival post-infection with *P. entomophila*, but often larval diets are important for post-infection survival when larvae are infected (e.g. Lee et al. 2006; Povey et al. 2014; Cotter et al. 2019; Wilson et al. 2020). These results suggest that for *P. entomophila* infection, only low P:C diets fed to adults, not larvae, reduce post-infection survival.

While not tested here, the lack of change on adult survival post-infection may have been due to compensatory feeding (e.g. Raubenheimer & Simpson, 1993; Ponton et al., 2013; Nestel et al., 2016). Adults that developed on low P:C may have been able to increase their feeding prior to or after infection, and were able to consume enough nutrients to survive infection to the same extent as adults that developed on higher P:C. With *P. entomophila* infection, this may have been due to increased consumption of protein, as survival on low P:C diets as adults was low. More tests are required to determine which component of diet was limiting (see section 6.2.3.2). Further studies applying a food choice component after larval macronutrient changes and after infection are required to understand whether larval diets alter adult compensatory mechanisms in combination with infection. I also did not measure whether combinations of larval and adult diets affect these responses further, requiring further testing to understand whether certain larval P:C diets become more optimal depending on the adult environment.

Results from Chapter 3 indicate that larval dietary P:C does not alter adult injury or infection responses. In contrast, larval feeding has been found to alter adult desiccation, temperature of starvation resistance (Andersen et al., 2010; Pascacio-Villafán et al., 2016; Davies et al., 2018). For example, when tested with two diets with changes in P:C and calories, higher larval P:C increased adult desiccation and heat resistance, but slowed cold stress recovery in *D. melanogaster* (Andersen et al., 2010). Instead, desiccation together with starvation resistance was highest on intermediate larval P:C in *D. melanogaster* (Pascacio-Villafán et al., 2016). These studies suggest different diets may be more optimal for different stress responses in adulthood, and that larval feeding may affect adult traits

depending on the type of stressor. As these studies only apply intense stressors for a short time and do not measure life-history traits post-stressor (Andersen et al., 2010; Pascacio-Villafán et al., 2016; Davies et al., 2018), additional studies should combine lifetime life-history measures to determine whether short-term temperature, desiccation or starvation stressors affect lifespan and reproduction, or whether larval feeding has no effect on these responses, as found here with infection.

6.2.3.2 Infection does not alter diet-mediated reproduction patterns:

Similar to previous studies, *P. entomophila* infection reduced reproduction (reviewed in Schwenke et al., 2016). However, infection did not change the overall relationships of macronutrient effects on reproduction, as seen in previous studies altering diet and infection status (Stahlschmidt et al., 2013; Kutzer & Armitage, 2016b; Kutzer et al., 2018). Infection had no effect on early-life reproduction in adults, irrespective of larval or adult feeding. One contrasting pattern to this general finding was that infection did significantly alter larval and adult P:C effects on egg production ageing, however the overall patterns with P:C were broadly similar, suggesting infection did not substantially alter diet effects on reproductive ageing.

In contrast to the early-life reproduction results here, a study applying *Pseudomonas aeruginosa* oral infection with two P:C diets found infection increased early-life egg production, but only for individuals on the higher P:C diet (Hudson et al., 2019). This was suggested to be due to terminal investment in terms of fecundity compensation, which was only present when protein was less limiting (Hudson et al., 2019, terminal investment reviewed in Clutton-Brock 1984; Kutzer and Armitage 2016a). The lack of a similar response in increased egg laying on higher P:C diets here with systemic *P. entomophila* infection suggests that fecundity compensation may be dependent on the pathogen, as seen previously in *D. melanogaster* using two pathogens and diets (Kutzer & Armitage, 2016b). As the method of infection also differed, further study is required to understand how

infection delivery affects infection patters when diets are altered (see also 6.2.4). By applying a range of P:C diets, where previously only two diets have been used (Kutzer & Armitage, 2016b; Hudson et al., 2019), here I show that infection did not alter diet-mediated reproductive patterns.

6.2.3.3 Infection altered diet mediated lifespan patterns without affecting reproduction:

As infection altered the dietary patterns of survival outcomes post-infection, but had no effect reproduction patterns, these results highlight that when diets are altered, infection can affect an important life-history trait of survival without affecting another, reproduction. The apparent diet-mediated lifespan-reproduction trade-off may have been decoupled with infection, where at low P:C, allocation of nutrients to competing life-history traits of reproduction, survival and responding to infection may have been limited, resulting in low lifespan but relatively high reproduction. Infection still came at a cost to lifetime survival, and potentially due to this lower survival, also to lifetime reproduction, as infected individuals had lower lifetime egg production when lifespan was not accounted for. This suggests that infection still causes trade-offs to lifetime traits, however at lower P:C diets, survival costs are more affected than reproduction with *P. entomophila*.

Resistance to infection has been thought of as a costly trait that trades-off with other life-history traits (reviewed in Zuk & Stoehr, 2002; Ponton et al., 2013). Tolerance has been suggested to limit fitness costs of responding to pathogens and so are not associated with fitness costs (see e.g. Roy & Kirchner, 2000; Howick & Lazzaro, 2014). In this *D. melanogaster – P. entomophila* host-pathogen pair, diet did not appear to alter resistance but instead disease tolerance was higher on higher P:C (Chapter 5), suggesting that at higher P:C, more resources may have been allocated to tolerance, and so higher survival, and reproduction could be maintained at a similar high level as in uninfected individuals. However, further experiments are required to determine whether diet increased tolerance due to the

low sample sizes of infected flies with quantifiable bacterial growth. Infected individuals did not increase their P:C intake (Chapter 4), and therefore I did not find evidence that individuals ate more of a given diet to potentially use in response to infection. Further tests are required to determine whether a longer feeding time, no prior starvation, or different diet pairs confirm these results. Similar to our survival results, some costs of infection have only been found with starvation (Wilson, 2005) or when diets are limited (Love et al. 2008, but see Stahlschmidt et al. 2013). In general, to understand how immune challenges affect life-history traits, it has been suggested that ecologically relevant conditions should be incorporated in studies (Zuk & Stoehr, 2002). The results in this thesis demonstrate the need to include diets ranging in P:C in infection studies measuring life-history traits.

Depending on the pathogen, caloric value of diets can influence post-infection survival, resistance or tolerance (Ayres & Schneider, 2009). Other nutrients may affect these processes as well depending on the pathogen, for example carbohydrates (Mason et al., 2014) or lipids (Adamo et al., 2010). In terms of studies manipulating both P:C ratios and calories, previous GF studies in insects have shown that time to death post-infection of *Spodoptera littoralis* caterpillars was more associated with protein, not carbohydrate, intake (Cotter et al., 2019). However, without a live infection, carbohydrate content as well affected measures of survival to pupation and immune measures in *S. littoralis* (Cotter et al., 2011).With infection, several immune responses peaked at different points in the macronutrient space (Cotter et al., 2019). Therefore, protein intake by itself does not explain all responses with infection. Future studies using *P. entomophila* should include GF approaches and lifetime measures of survival and reproduction post-infection to determine which component of P:C diets affects these lifetime trade-offs measured here, and whether they are present at all caloric values of diet. Additionally, these approaches may uncover which nutrient or component of diet is the possible limiting nutrient with *P. entomophila* infection (reviewed for

reproduction in Schwenke et al., 2016), as higher P:C, so either increasing protein, or lower carbohydrate, or their interaction increased post-infection survival.

As discussed earlier (see section 6.2.1.2), although survival and reproduction did not peak at the same diet, this does not necessarily indicate a trade-off, and may be due to constraints of nutrients in diets (reviewed in Moatt et al., 2020). A specific nutrient may be required for multiple life-history traits, and therefore its level of availability can influence life-history trade-offs with infection (reviewed in Zuk & Stoehr, 2002; Schwenke et al., 2016). In addition, different measures of the immune response have been found peak at different P:C ratios, suggesting trade-offs between different components of the immune response (Cotter et al., 2011, 2019). Here, higher P:C diets may have been better for tolerating *P. entomophila* infection, and higher P:C increased survival, and reproduction. Low P:C decreased survival, suggesting protein may be a limiting nutrient with *P. entomophila* infection. Protein is also required for egg production in female *D. melanogaster* (Wheeler, 1996; Mirth et al., 2019), and therefore individuals on higher P:C may have been able to invest resources into disease tolerance and reproduction simultaneously. Repeating these lifetime measures in *D. melanogaster* but using a pathogen where lower P:C increases survival, for example *Micrococcus luteus* (Ponton et al., 2020), *P. aeruginosa* or *Staphylococcus aureus* (Lee et al., 2017), would allow for estimations of how these life-history trade-offs change when post-infection survival and reproduction are maximised at more extremes of the P:C intakes. One potential outcome would be that infected individuals would have higher lifetime survival on low P:C diets, however lifetime reproduction would be greatly reduced on higher P:C diets, as infected individuals have low survival and therefore less time to reproduce.

6.2.3.4 How would male *D. melanogaster* respond to changes in P:C and infection with *P. entomophila*?

One additional future area of study is repeating these experiments in male flies, as for logistical reasons, only female flies were used here. There are several

reasons why diet and infection treatments may interact differently in female and male flies, therefore making them an ideal system to study such interactions. First, male and female *D. melanogaster* respond to the same DR treatments differently, where females generally respond more to a given level of restriction (e.g. Magwere et al., 2004). From meta-analyses, such sex-specific differences with diet are apparent across taxa (Nakagawa et al., 2012; Moatt et al., 2016). Second, there is considerable variation in how males and females respond to infection (reviewed in Zuk & Stoehr, 2002; Klein & Flanagan, 2016; Metcalf et al., 2019; Belmonte et al., 2020). Third, diet may affect this variation in infection survival further, for example in *G. texensis* field crickets, females on low P:C diets had higher survival post-infection with *S. marcescens,* whereas male crickets had higher survival on high P:C diets (Kelly & Tawes, 2013). Therefore, further studies should implement similar experiments as used here to explore whether diet and infection interactions change life-history trade-offs differently in males and females.

In male *D. melanogaster*, reproduction appears to be more determined by the carbohydrate content of diet, where a measure of male reproduction peaked at low P:C diets (Jensen et al., 2015). In females, reproduction instead peaked at a higher P:C diets (Jensen et al., 2015), potentially due to requiring protein for egg production (reviewed in Wheeler, 1996; Mirth et al., 2019). As male flies may not have such a high requirement for protein, experiments using male flies may have different patterns of survival post-infection when infection experiments implement pathogens, where survival post-infection is associated with higher P:C in females, for example with *P. entomophila*. However, studies in *D. melanogaster* highlight pathogen-specificity in the direction of the survival bias post-infection (Belmonte et al., 2020). As this has not been tested with *P. entomophila*, it is difficult to predict what the exact survival outcomes would be. To understand how these patterns change infection outcomes, studies should apply different pathogens where post-infection is associated with higher or lower P:C, and combine these with pathogens where the survival direction between males and females differs.

Oral infection with *P. entomophila* is suggested to damage the gut (reviewed in Dieppois et al., 2015), and gut integrity with age in female *D. melanogaster* is maintained longer on simultaneous lower caloric and P:C diets, where male flies do not see such an improvement with the same diet manipulation (Regan et al., 2016). Therefore, for *P. entomophila* specifically, further tests should use oral infection in both male and female *D. melanogaster* to study whether these differences in gut ageing with diet affect infection outcomes. One potential outcome for such tests could be that increasing dietary P:C has less of an effect in *P. entomophila* oral infection survival outcomes in males, as changes in diet does not affect their gut epithelium to the same extent as in female flies and therefore diet may not affect their survival to the same extent as females with a gut infection. In general, more infection studies using both males and females when diets are altered are required to understand this potentially complex relationship and to determine how general diet effects on post-infection traits are.

6.2.4 Host-pathogen pair specificity in diet effects - diet alters *D. melanogaster* survival outcomes and disease tolerance, but does not alter food choice with *P. entomophila:*

With a great variety of potential pathogens, individuals are not equally good at responding to all types of infections (reviewed in Zuk & Stoehr, 2002). In addition, life-history trade-offs post-infection may depend on many factors including the host species and the specific type of infection (reviewed in Zuk and Stoehr 2002; Schoenle et al. 2018). *P. entomophila* infection has been extensively studied in *D. melanogaster* (e.g. Bou Sleiman et al., 2015; Dieppois et al., 2015; Vijendravarma et al., 2015; Loch et al., 2017), however this pathogen has not been used in studies measuring effects of diets ranging only in P:C on infection and with a genetically heterogeneous population of flies. In this thesis, I showed that only higher P:C content of adult diet increases *D. melanogaster* survival after infection with *P. entomophila* due to increased tolerance, however post-infection, individuals do not choose more of a diet associated with higher survival than control or injured individuals (but see discussion above for limitations in these studies). For other

traits, although there were some small changes across diets with infection, infection did not substantially affect negative geotaxis, ageing in egg laying, or the interactions between larval P:C and egg laying traits.

P. entomophila has been used in two other insect studies focusing on diet effects on survival (Kutzer et al. 2018; Sieksmeyer et al. 2019). Also in *D. melanogaster*, a reduction in P:C and calories reduced survival post-infection, however changes in bacterial load were not measured due to a high number of individuals dying with reduced P:C and calories (Kutzer et al., 2018). In contrast to findings in *D. melanogaster*, P:C had no effect on post-infection survival in *Blatta orientalis* cockroaches, but infected individuals reduced their food intake and changed their food choice to a higher P:C ratio a day post-infection (Sieksmeyer et al., 2021). The results of this thesis together with these two studies suggest that the same pathogen, *P. entomophila*, with the same infection method of systemic infection, had contrasting effects on survival and food choice depending on the host (Kutzer et al., 2018; Sieksmeyer et al., 2021).

There are several potential reasons for these different results depending on the host. First, these effects may be due to the host nutritional environment, for example as suggested for *B. orientalis,* from their potential adaptations to detoxification or the range of diet sources affecting innate immunity (Sieksmeyer et al. 2019). However, *D. melanogaster* feed on rotting fruit, which are associated with high numbers of potentially harmful pathogens (reviewed in Markow, 2015; Mistry et al., 2016). Second, *P. entomophila* is proposed to be an opportunistic soil entomopathogen (reviewed in Dieppois et al., 2015), and so these differences may also be due to diverse effects of how *P. entomophila* interacts with the host biology or their within-host nutritional environment. Future studies should incorporate lifetime measures in *B. orientalis* or other insect hosts to allow for easier comparisons to the results presented here and to determine how the same pathogen affects diet-mediated patterns across host species.

It is unclear why *P. entomophila* affects host food choice in *B. orientalis* but not in *D. melanogaster*, especially as the change in food choice in *B. orientalis* was

not associated with an increase in survival (Sieksmeyer et al., 2021), which is often found with other pathogens (reviewed in Hite et al., 2020). As only two P:C diets were used to measure survival (Sieksmeyer et al., 2021), more tests in *B. orientalis* are required to determine whether changes in post-infection survival were present when multiple P:C diets are used. One other potential reason that *P. entomophila* had no effect on food choice as measured here, is that in some combinations of host-pathogen pairs, infection may not change food choice, which requires further testing with additional host-pathogen pairs.

An additional reason for this pattern is the estimated diluted dose. When two septic infection doses of *P. entomophila* were used to infect *B. orientalis,* only the higher infection dose resulted in a change in food choice behaviour (Sieksmeyer et al., 2021). Therefore, higher doses should be used in *D. melanogaster* to determine whether similar dose effects occur in another host. Dose effects affecting diet patterns have been found in another host-pathogen pair, where with increasing *Xenorhabdus nematophila* bacterial injection dose in *S. littoralis* caterpillars, P:C effects on post-infection speed of death and bacterial load increased (Wilson et al., 2020). In addition, as septic infection was used here, however *P. entomophila* is suggested to infect flies when flies eat infected material containing the bacteria (reviewed in Dieppois et al., 2015), further experiments should compare septic and more natural oral infections using this pathogen.

Outside of *P. entomophila* infection, there is increasing evidence for diet having differential effects depending on the host or pathogen applied (see e.g. Becker et al. 2015; Pike et al. 2019; Roberts and Longdon 2020). More specifically for *D. melanogaster,* depending on the pathogen, higher P:C is associated with higher (Le Rohellec & Le Bourg, 2009; Kutzer et al., 2018), or lower post-infection survival (Lee et al., 2017; Ponton et al., 2020), or even no change in survival with *Lactococcus lactis* (Kutzer et al. 2018). From these studies where diet affects post-infection survival, infection with a particular pathogen has been linked to increased resistance on the diet with higher post-infection survival (Lee et al., 2017; Ponton et al., 2020). However, in one study both calories and P:C were altered (Lee et al.,

2017), or bacterial loads were not measured (Ponton et al., 2020). The results from this thesis highlight a case of increased tolerance with diet with another pathogen, where only P:C and not calories were manipulated and where bacterial loads were measured.

Although it is unclear why changes in diet are associated with changes in resistance or tolerance, one potential reason is that the host and pathogen compete for resources (Ponton et al., 2013; Cressler et al., 2014) and different pathogens may compete for different resources, or be affected by the presence of different resources differently (see e.g. Wilson et al. 2020). Additionally, as different host immune responses are more optimised at different diets (e.g. Lee et al., 2006; Cotter et al., 2011, 2019; Povey et al., 2014), infection outcomes may be altered due to immune responses or tolerance mechanisms that hinder the growth of the pathogen being higher on the specific diet. Overall, more studies applying different pathogens and repeating the experiments here are required to uncover why *D. melanogaster* and other hosts have such wide ranging infection outcomes and host responses depending on the pathogen.

6.3 Conclusions:

The work in this thesis has demonstrated for the first time that DR in the form of macronutrient ratio manipulation still extends lifespan with additional stressors of injury and infection. Other common DR responses remained with these additional stressors, as regardless of injury and infection, reproduction reduced and ageing was delayed. Overall, these results therefore do not provide evidence to support the NRH (Adler and Bonduriansky 2014) and provide more support for the RRH (Shanley & Kirkwood, 2000). While further studies focusing on the various evolutionary hypotheses of DR are required to understand why DR increases lifespan (Shanley and Kirkwood 2000; Fanson et al. 2012; Adler and Bonduriansky 2014; Speakman 2020), these results highlight that DR responses can be present in more stressful environments than the benign laboratory environment. I also found that larval P:C manipulation did not affect adult life-history traits to a great degree, although intermediate larval P:C diets increased adult reproduction measures. Such carry-over effects from larval feeding should be considered when designing insect studies focusing on only changing the adult dietary environments.

Although the lifespan extension of DR remained with infection, I found that with *P. entomophila* infection, low P:C diets in particular were detrimental for lifespan, and low P:C may be associated with lower disease tolerance but not resistance, requiring further study. Therefore, although DR responses are apparent with additional stressors, some diets may be associated with lower lifetime post-infection survival, and future infection studies focusing on trade-offs should incorporate various diets to measure how life-history trade-offs change with diet availability and changes in nutrients. Larval dietary P:C did not alter any adult trait post-infection, potentially due to compensatory feeding, suggesting the current environment is more important for determining infection outcomes.
D. melanogaster infected with *P. entomophila* had no change in food choice, which suggests that changes in food choice do not appear with all host-pathogen pairs. Overall, these results provide further evidence of host-pathogen pair specificity in effects of diet on infection outcomes. In addition, these results highlight the

complexity between the effects of diet on infection outcomes, where trade-offs between traits with infection are complex, depend on the host-pathogen pair, and require further testing to understand which component or components of diet are affecting these patterns.

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Appendices:

(Entire Appendix A was written and all work described was completed by Fergal M. Waldron and Katy M. Monteith.)

7.1 Appendix A: Outcrossed DGRP population - A genetically diverse laboratory population resource for *Drosophila melanogaster* studies

The outcrossed DGRP population was founded on 15/10/14, and is derived from 113 inbred DGRP lines sampled from a wild population in Raleigh, NC, USA (Mackay et al., 2012).

The outcrossed DGRP population was founded with genetic contributions from the following 113 DGRP lines; RAL-28, RAL-31, RAL-48, RAL-49, RAL-57, RAL-59, RAL-69, RAL-75, RAL-83, RAL-91, RAL-93, RAL-101, RAL-129, RAL-138, RAL-149, RAL-153, RAL-158, RAL-189, RAL-195, RAL-208, RAL-217, RAL-228, RAL-237, RAL-239, RAL-280, RAL-287, RAL-288, RAL-301, RAL-303, RAL-304, RAL-306, RAL-309, RAL-310, RAL-317, RAL-321, RAL-324, RAL-348, RAL-350, RAL-352, RAL-354, RAL-358, RAL-360, RAL-361, RAL-365, RAL-366, RAL-373, RAL-375, RAL-377, RAL-379, RAL-380, RAL-381, RAL-382, RAL-386, RAL-390, RAL-392, RAL-395, RAL-397, RAL-399, RAL-405, RAL-406, RAL-409, RAL-426, RAL-427, RAL-437, RAL-439, RAL-443, RAL-486, RAL-491, RAL-492, RAL-502, RAL-508, RAL-509, RAL-517, RAL-528, RAL-530, RAL-535, RAL-555, RAL-559, RAL-563, RAL-566, RAL-575, RAL-584, RAL-589, RAL-627, RAL-630, RAL-634, RAL-703, RAL-712, RAL-716, RAL-732, RAL-765, RAL-774, RAL-776, RAL-786, RAL-796, RAL-805, RAL-808, RAL-818, RAL-820, RAL-821, RAL-822, RAL-832, RAL-852, RAL-853, RAL-855, RAL-859, RAL-879, RAL-882, RAL-884, RAL-897, RAL-907, RAL-908, RAL-913.

To maximise the genetic contribution of each of the founder lines to the final outcrossed population, initial pairwise crosses between randomly selected population founder lines were carried out. The offspring from these pairwise crosses were then pooled into a population cage for the 1st generation of outcrossing. Whilst a minimum of 57 pairwise crosses would encompass inclusion of all 113 founder lines, 100 pairwise crosses were carried out as a precautionary measure against a number of crosses failing to produce offspring (an upper limit of 100 was dictated by feasibility). Pairwise crosses were set up using two virgin

females crossed to two males. All virgin females and males were age-matched controlled (1-6 days old when crosses were set up). Pairwise crosses were set up in standard Lewis medium containing vials and placed at 25°C for 5 days after which adults were removed.

For pairwise crosses, DGRP outcrossed population founder lines were randomly selected to contribute females or males for the following 100 crosses (scheme is "2 virgin females from line" x "2 males from line"): RAL-390 x RAL-381, RAL-280 x RAL-301, RAL-913 x RAL-365, RAL-796 x RAL-712, RAL-589 x RAL-49, RAL-350 x RAL-382, RAL-853 x RAL-158, RAL-288 x RAL-855, RAL-49 x RAL-366, RAL-303 x RAL-908, RAL-101 x RAL-303, RAL-712 x RAL-426, RAL-321 x RAL-732, RAL-377 x RAL-101, RAL-380 x RAL-879, RAL-820 x RAL-324, RAL-882 x RAL-535, RAL-439 x RAL-634, RAL-83 x RAL-409, RAL-28 x RAL-75, RAL-409 x RAL-832, RAL-879 x RAL-237, RAL-237 x RAL-239, RAL-443 x RAL-776, RAL-908 x RAL-627, RAL-59 x RAL-584, RAL-365 x RAL-796, RAL-634 x RAL-405, RAL-392 x RAL-852, RAL-129 x RAL-350, RAL-317 x RAL-306, RAL-427 x RAL-528, RAL-373 x RAL-502, RAL-386 x RAL-28, RAL-304 x RAL-392, RAL-774 x RAL-555, RAL-306 x RAL-386, RAL-310 x RAL-309, RAL-832 x RAL-287, RAL-405 x RAL-280, RAL-57 x RAL-774, RAL-627 x RAL-228, RAL-397 x RAL-821, RAL-348 x RAL-492, RAL-437 x RAL-443, RAL-91 x RAL-31, RAL-352 x RAL-575, RAL-301 x RAL-390, RAL-48 x RAL-897, RAL-575 x RAL-808, RAL-426 x RAL-373, RAL-375 x RAL-195, RAL-31 x RAL-59, RAL-897 x RAL-310, RAL-239 x RAL-486, RAL-287 x RAL-805, RAL-584 x RAL-765, RAL-381 x RAL-149, RAL-93 x RAL-703, RAL-379 x RAL-517, RAL-821 x RAL-630, RAL-189 x RAL-853, RAL-399 x RAL-360, RAL-907 x RAL-217, RAL-535 x RAL-786, RAL-195 x RAL-395, RAL-852 x RAL-913, RAL-502 x RAL-818, RAL-361 x RAL-375, RAL-138 x RAL-491, RAL-808 x RAL-93, RAL-517 x RAL-208, RAL-153 x RAL-189, RAL-149 x RAL-352, RAL-732 x RAL-509, RAL-818 x RAL-563, RAL-630 x RAL-57, RAL-395 x RAL-380, RAL-358 x RAL-822, RAL-765 x RAL-406, RAL-703 x RAL-859, RAL-406 x RAL-153, RAL-508 x RAL-379, RAL-716 x RAL-427, RAL-509 x RAL-358, RAL-555 x RAL-48, RAL-360 x RAL-321, RAL-786 x RAL-69, RAL-855 x RAL-354, RAL-559 x RAL-437, RAL-563 x RAL-361, RAL-158 x RAL-559, RAL-805 x RAL-884, RAL-208 x

RAL-566, RAL-492 x RAL-397, RAL-382 x RAL-399, RAL-75 x RAL-508, RAL-884 x RAL-138, RAL-530 x RAL-83, RAL-69 x RAL-348. Offspring from pairwise crosses were collected 28 days after parents were removed and pooled into a large *Drosophila* population cage, for the 1st generation of outcrossing and subsequent embryo collection.

For this, and each subsequent generation of outcrossing, the outcrossed DGRP population is maintained employing a method used to maintain constant larval densities (223 ± 14.3 (95% CI)) in stock bottles (Clancy & Kennington, 2001). Briefly, this method involves populating a large *Drosophila* cage with thousands of flies on the day 1, providing these with fruit juice (grape/apple) agar plates for embryo laying. After a 24 hr habituation period, agar plates are replaced (day 2). On the day 3, agar plates are recovered and embryos are collected from the surface. Using PBS and a brush, concentrated egg/PBS solutions are prepared, and these are squirted on the surface of Lewis media in bottles. This process is typically carried out every 20-25 days. The outcrossed DGRP populations is maintained at a density of 20-25 bottles (20 bottles maintains the population at >4000 individuals).

7.2 Appendix B: Chapter 2 supplement

7.2.1 Supplementary methods:

7.2.1.1 Negative geotaxis (NG) assay:

This assay quantifies the climbing response of flies in terms of distance or speed, following Arking and Wells (1990). A rubber band was tied 4 cm from the bottom around an empty vial. After the fly was tipped into this vial and blocked with a cotton bud, the vial was tapped down three times on a corkboard. The timer was started on the last tap and stopped once the fly fully crossed the line. After the test, the fly was transferred to a new food vial. An upper limit of 60 seconds was set as some flies did not climb or cross the line. One vial was used per fly to avoid confounding effects of reusing vials (Nichols et al., 2012) or possible spread of infection. Due to time of day effects (Gargano et al., 2005), testing order was reversed each week. If the fly did not touch the bottom of the vial, or if the timer was stopped incorrectly, a second trial was completed. Due to the number of failed tests where the fly did not cross the line (43% of 5,117 tests), negative geotaxis scores were analysed as a binomial variable for passing (1) or failing (0) the test in 60 seconds.

7.2.1.2 Statistical methods:

7.2.1.2.1 Survival:

We used the R Survminer package (Kassambara & Kosinski, 2018) to graph Kaplan-Mayer curves individually for each stress treatment with diet as a factor. We first analysed the survival data with a Cox proportional hazards model using the R Survival package (Therneau, 2015). The model included protein content, its squared term, stress treatments and their interactions as fixed effects. The assumptions of a Cox proportional hazards model were violated (Therneau, 2015, cox.zph function global term Chi squared = 95.26, p = <0.001). Predicted risk ratios

for each diet and stress treatment were calculated using the predict function for the Cox proportional hazards model.

As our survival data did not follow the assumptions of a Cox proportional hazards model, therefore we used an event history model where survival was analysed as a binomial trait, with each day a fly scored as a 0 for being alive and 1 for dead, following Moatt et al. (2019). We used the R package MCMCglmm (Hadfield, 2010) to model survival as a binomial variable with a categorical model. The model contained the fixed effects of stress treatment, protein content and its squared term (to model non-linear effects) and their interaction. Censored flies were included in the analysis (27 individuals, so 4.5% of the total), scoring a 0 until the day of censoring. A random effect of individual identity was included to account for repeated measures on the same individual and a random effect of experimental day was added to account for variation in survival across days. Parameter expanded priors were placed on all random effects ($V = 1$, $nu = 1$, $alpha.mu = 0$, $alpha.V = 1000$). The residual variance was fixed to 1, as it is inestimable in a binomial model. The model was run for 5,200,000 iterations, with a burnin of 1,200,000 iterations and a thinning interval of 4,000 iterations to minimise autocorrelation. Autocorrelation was checked from plots of the posterior distribution of all estimates for this and all subsequent models.

We also analysed lifespan to confirm the results of the survival analysis. Lifespan, the number of days an individual survived, was analysed using a generalised linear model with MCMCglmm. Censored flies were removed from the analysis. A Poisson family error distribution was assumed and the model was run for 65,000 iterations with a thinning interval of 50 iterations and a burnin of 15,000 iterations to minimise autocorrelation. Protein content, its squared term, stress treatments and their interactions were included as fixed effects. An inverse Gamma prior was placed on the residual variance ($V = 1$ and $nu = 0.002$).

Similar models were used to analyse the effect of diet on individuals dying before 10 days post-infection treatment and on individuals that survived at least to this time point. First, changes in effects of diet on mortality was analysed using an

event history binomial model with only infected flies, as only a few injured or unstressed control flies died before 10 days post-stress treatments (Appendix B, Table S2.3). All flies were categorised as dying before or after 10 days post-infection, and this category as well as its interactions with protein and its squared term were added as fixed effects and the model was run for 2,600,000 iterations, with a burnin of 600,000 iterations and a thinning interval of 1,000 iterations. As a Cox proportional hazards model on the infected flies dying before 10 days post-infection (and other flies included as censored data points on day 10) did not meet the assumptions of proportional hazards (cox.zph function global term Chi squared = 6.21, p = 0.05), and the event history binomial model failed to run with this sample size (not shown), only lifespan data was analysed. This model was ran for 13,000 iterations, with a burnin of 3,000 iterations and a thinning interval of 10 iterations. For flies that survived this initial higher mortality of infected flies, again the assumptions of proportional hazards was not met (cox.zph function global term Chi squared = 25.59, p = 0.001), and an event history binomial model (ran for 2,600,000 iterations, with burnin of 600,000 iterations and a thinning interval of 2,000 iterations) and a linear lifespan model were ran (ran for 2,600,000 iterations, with burnin of 600,000 iterations and a thinning interval of 2,000 iterations).

7.2.1.2.2 Reproduction:

Lifetime reproduction was measured as the sum of all eggs counted per female over her life. The effect of stress treatment, protein content, its squared term and their interactions were analysed using a MCMCglmm model with a Poisson error distribution. The model was run for 130,000 iterations, with a burnin of 30,000 iterations and a thinning interval of 100 iterations to minimise autocorrelation. An inverse Gamma prior was placed on the residual variance ($V = 1$ and $nu = 0.002$). To remove the effect of lifespan on reproduction, the same model with the effect of mean centered lifespan for each fly was analysed separately, except with 650,000 iterations, a burnin of 150,000 iterations and a thinning interval of 500. As an additional analysis to remove the effect of lifespan on reproduction and to compare our data with other studies using measures of

early reproduction, early egg production was analysed separately. Egg counts from experimental day 2 (day after stress treatment) to day 7 were considered, as the first day egg counts were very low and were very similar across diets (Figure S1). Only individuals which lived to day 7 were considered. A MCMCglmm model with a Poisson error distribution was run with 260,000 iterations, a burnin of 60,000 iterations and a thinning interval of 200 iterations. An inverse Gamma prior was placed on the residual variance ($V = 1$ and $nu = 0.002$). The effect of stress treatment, protein content and its squared term were included in the model.

7.2.1.2.3 Reproductive ageing:

To investigate reproductive senescence, daily egg counts were analysed using MCMCglmm with a Poisson error distribution. When egg counts changed from daily to every second day counting, all values that correspond to eggs produced over two days were divided by two and rounded down to the nearest integer. Fixed effects included stress treatment, protein content and age (in days) and their squared terms, and all interactions. Mean centred lifespan was included as a fixed effect to control for selective disappearance (Van de Pol & Verhulst, 2006) and individual ID was included as a random effect to control for repeated measures on the same individual. Models were run for 2,600,000 iterations, with a thinning interval of 1,500 and a burnin of 600,000. A parameter expanded prior was used for the random effect of individual ($V = 1$, $nu = 1$, $alpha.mu$ = 0, $alpha.V = 1000$) and an inverse Gamma prior placed on the residuals ($V = 1$ and $nu = 0.002$).

7.2.1.2.4 Gut deterioration (smurf) assay:

A fly was scored as a smurf if it developed a non-disappearing blue body appearance (1 for smurf, 0 for no smurf) at any point during its life. This binomial variable was analysed with a categorical model using MCMCglmm, with only flies that survived at least 18 days (appearance of first smurf). This model included the fixed effects of stress treatment, protein content, its squared term and their interactions. Models were run for 19,500,000 iterations, with a thinning interval of

15,000 and a burnin of 4,500,000. The residuals variance was fixed to 1 as explained above.

7.2.1.2.5 Negative geotaxis (NG) assay:

We analysed the data from the negative geotaxis experiments as a binomial variable (1 for climbing 4 cm in 60 seconds, 0 for failing to do this) using a categorical family in MCMCglmm. Stress treatment, protein content and age and their squared terms, their interactions and mean centred lifespan were included as fixed effects and individual identity as a random effect. The model was run for 3,900,000 iterations, with a thinning interval of 3,000 and a burnin of 900,000. A parameter expanded prior was used for individual identity ($V = 1, nu = 1, alpha.mu = 0, alpha.V = 1000$) and the residual variance was fixed to 1 as explained above.

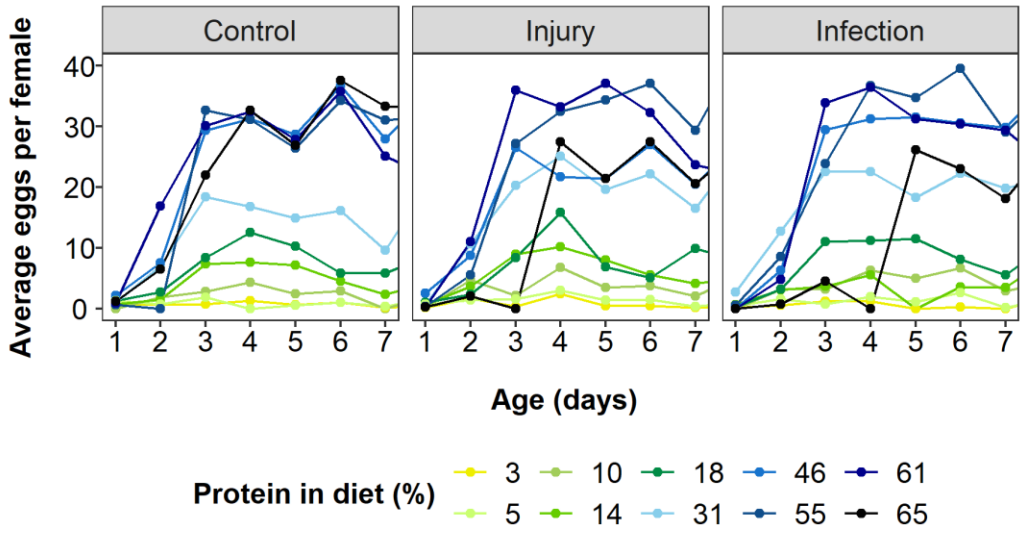

Figure S2.1: Average eggs per day produced in the first week for each protein restriction diet of flies infected with a bacterial pathogen ("Infection"), injured by a pinprick ("Injury") or with no treatment ("Control").

7.2.1 Supplementary results:

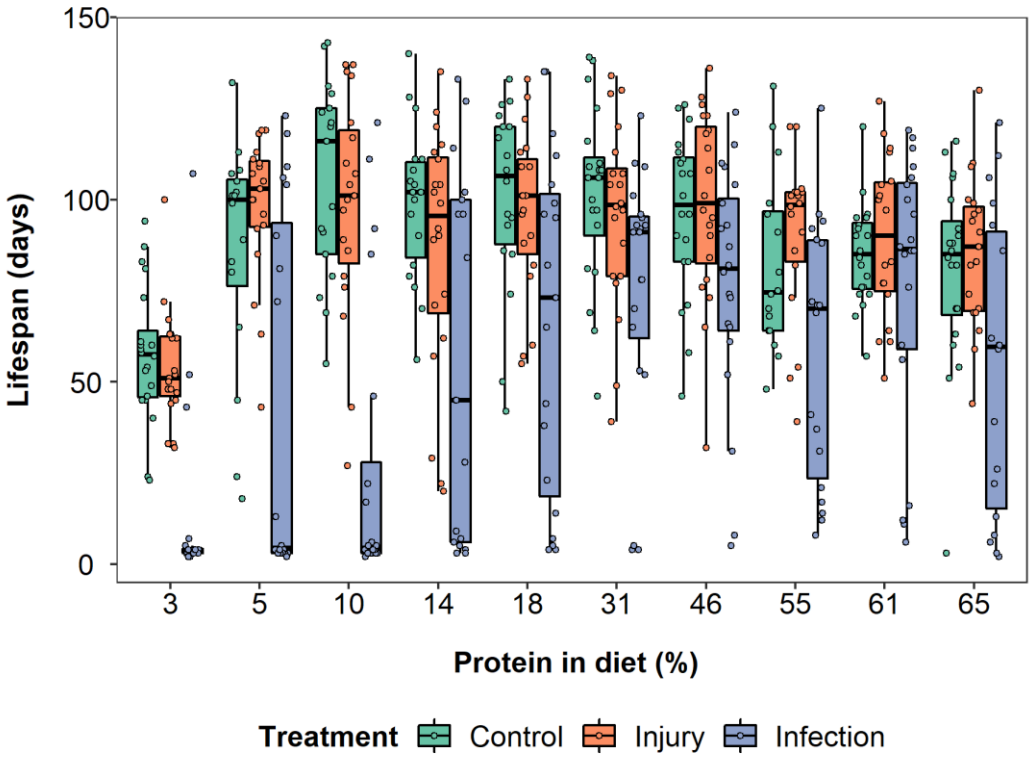

Figure S2.2: Effects of protein restriction on the lifespan of flies infected with a bacterial pathogen (blue bars and data points), injured by a pinprick (orange bars and data points) or with no treatment (green bars and data points). Data are observed lifespans (filled circles), where lines in the box plots indicate median lifespan (50% quantile), boxes are the interquartile range (25% to 75% quantiles) and whiskers are minimum or maximum quartiles (25% - 1.5 x interquartile range, 75% + 1.5 x interquartile range).

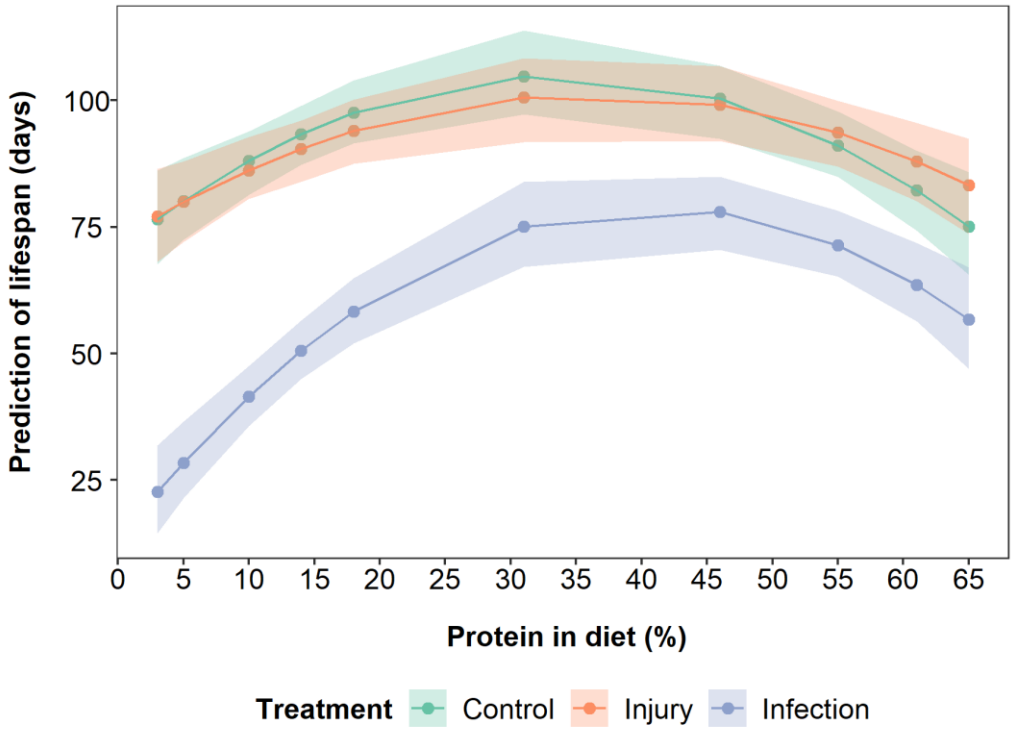

Figure S2.3: Model predictions of the effects of protein restriction on lifespan of flies infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines). Shaded areas are 95% 95% credible intervals. Protein and protein^2 are mean centered to standard deviation of 1.

Table S2.1: Model summary of effects of protein restriction and stress treatments on lifespan. Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded.

	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
Intercept	**104.76**	**97.22**	**113.77**	**1000**	**<0.001**
Injury treatment	-4.17	-15.32	7.55	1107	0.48
Infection treatment	**-29.83**	**-41.32**	**-17.72**	**1000**	**<0.001**
Protein	3.83	-0.16	-9.21	1000	0.09
Protein^2	**-15.79**	**-22.55**	**-8.90**	**1108**	**<0.001**
Injury:Protein	1.57	-5.97	7.50	1000	0.65
Infection:Protein	**14.31**	**7.66**	**20.99**	**1000**	**<0.001**
Injury:Protein^2	4.47	-5.33	14.45	1158	0.39
Infection:Protein^2	-4.44	-14.45	6.06	1000	0.40

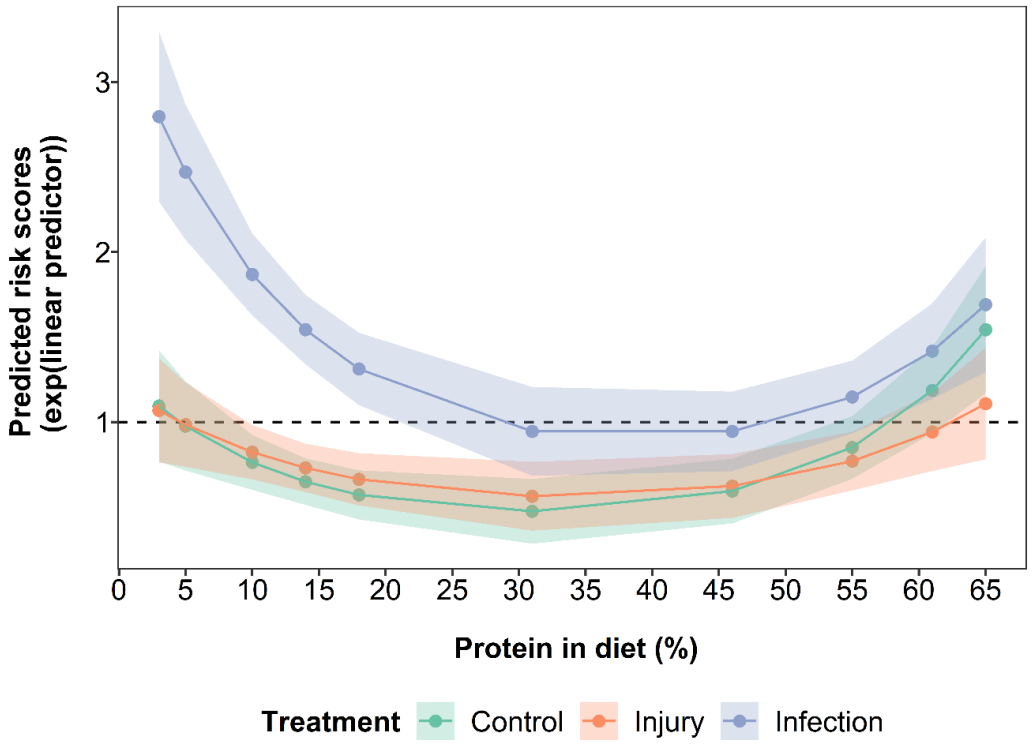

Figure S2.4: Model predictions for the effects of protein restriction on survival of flies infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines). $y = 1$ line shows no change in risk ratio, i.e. treatment would have no effect compared to baseline hazard. Shaded areas are 95% confidence intervals. Protein and protein^2 are mean centered to standard deviation of 1.

Table S2.2: Cox proportional hazard regression model summary of effects of protein restriction and stress treatments on survival (n = 600, number of deaths = 573, concordance = 0.662, R^2 = 0.142, Wald test = 97.98). Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level α = 0.05 are bolded.

	coef	exp(coef)	se(coef)	z	Pr (>|z|)
Injury treatment	0.17	1.19	0.20	0.84	0.40
Infection treatment	**0.69**	**1.99**	**0.21**	**3.36**	**<0.001**
Protein	-0.03	0.97	0.09	-0.29	0.77
Protein^2	**0.54**	**1.72**	**0.12**	**4.28**	**<0.001**
Injury:Protein	-0.06	0.94	0.12	-0.52	0.60
Infection:Protein	**-0.29**	**0.75**	**0.12**	**-2.43**	**0.01**
Injury:Protein^2	-0.18	0.83	0.18	-1.03	0.30
Infection:Protein^2	-0.07	0.93	0.18	-0.41	0.68

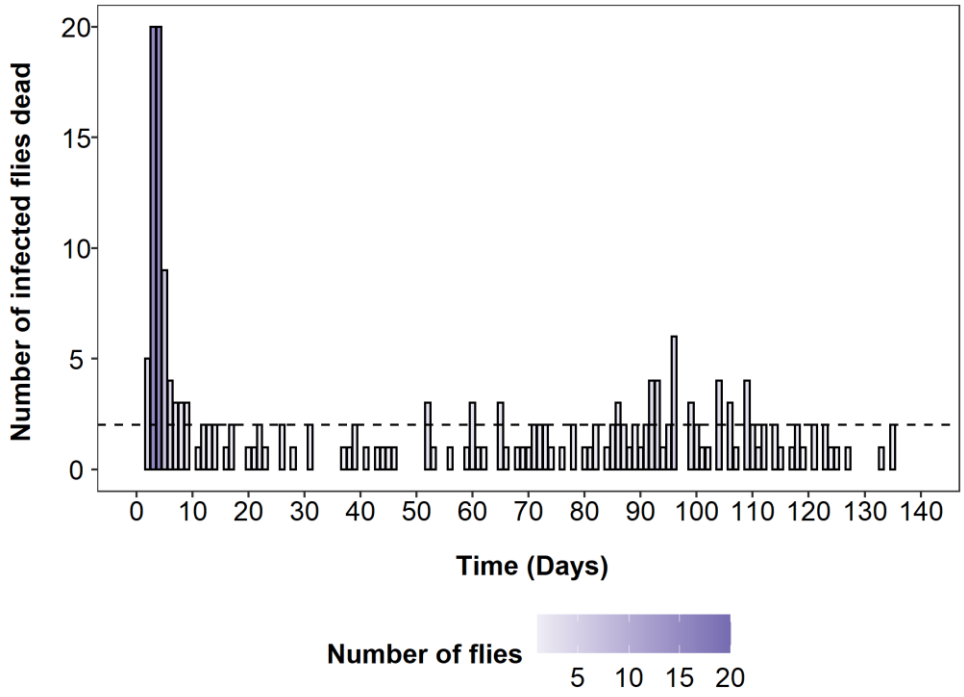

Figure S2.5: Number of flies dead post-infection treatment. The dotted line shows where the number of dead flies per day is 2.

Table S2.3: Sample sizes for flies when separated into early-mortality post-stress treatment (dying or going missing prior to day 10) and later-life mortality (dying or going missing after 10 days). All diet by stress treatment groups had an initial 20 flies each.

Stress treatment	Diet (protein %)	Number of flies dead or missing prior to day 10	Number of flies dead or missing after day 10
Control	3	0	20
	5	1	19
	10	1	19
	14	1	19
	18	1	19
	31	0	20
	46	0	20
	55	2	18
	61	0	20
	65	1	19
Injury	3	0	20
	5	0	20
	10	0	20
	14	0	20
	18	1	19
	31	0	20
	46	0	20
	55	0	20
	61	1	19
	65	0	20
Infection	3	17	3
	5	11	9
	10	13	7

	14	8	12
	18	4	16
	31	3	17
	46	2	18
	55	2	18
	61	1	19
	65	6	14

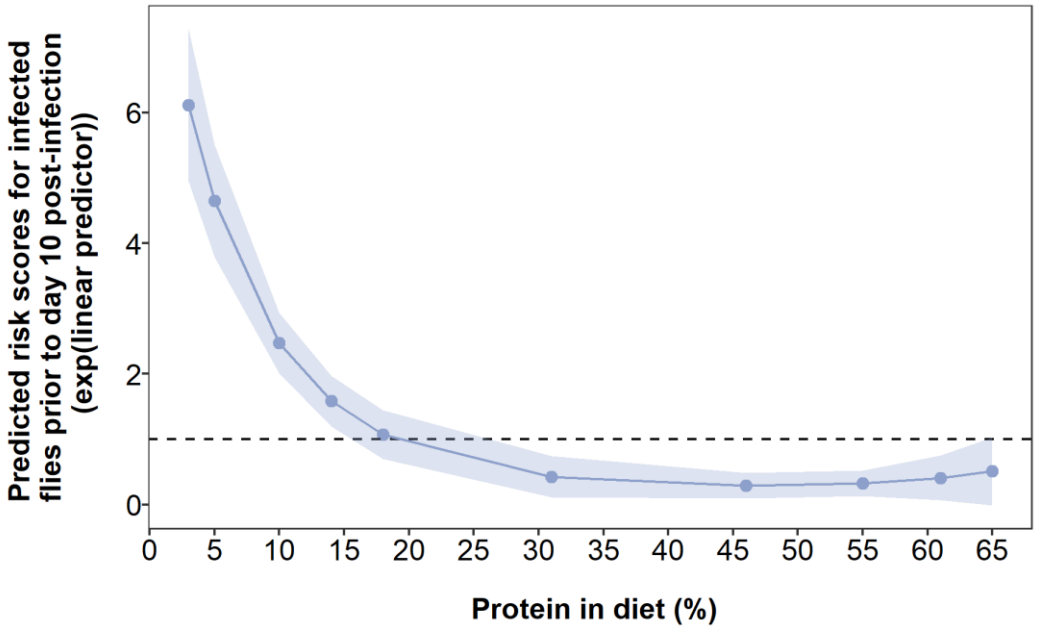

Figure S2.6: Model predictions for the effects of protein restriction on survival of flies infected with a bacterial pathogen prior to 10 days post-infection. $y = 1$ line shows no change in risk ratio, i.e. diet would have no effect compared to baseline hazard. Shaded areas are 95% confidence intervals. Protein and protein^2 are mean centered to standard deviation of 1.

TableS2.4: Cox proportional hazard regression model summary of effects of protein restriction on survival of infected flies prior to 10 days post-infection (n = 200, number of deaths = 63, concordance = 0.8, Wald test = 57.86). Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded.

	coef	exp(coef)	se(coef)	z	Pr (>|z|)
Protein	**-1.16**	**0.31**	**0.16**	**-7.10**	**<0.001**
Protein^2	**0.85**	**2.34**	**0.25**	**3.43**	**<0.001**

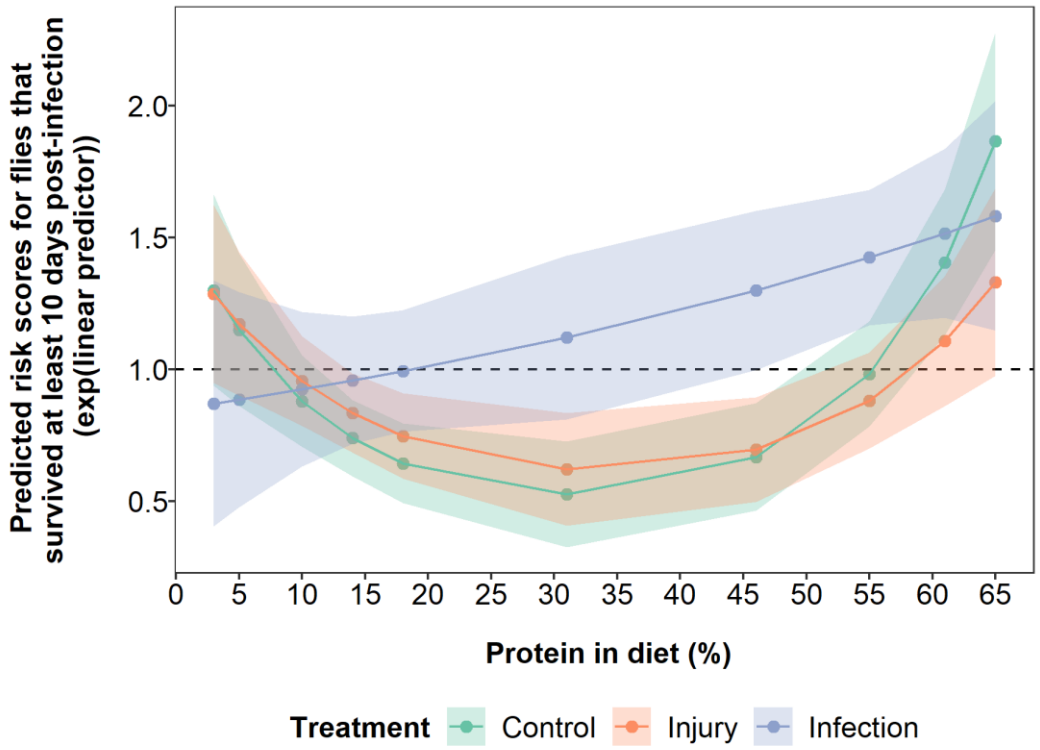

Figure S2.7: Model predictions for the effects of protein restriction on survival of flies infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines) for flies that survived at least 10 days post-stress treatment. $y = 1$ line shows no change in risk ratio, i.e. treatment would have no effect compared to baseline hazard. Shaded areas are 95% confidence intervals. Protein and protein^2 are mean centered to standard deviation of 1

Table S2.5: Cox proportional hazard regression model summary of effects of protein restriction and stress treatments on survival for flies that survived at least 10 days post-stress treatment (n = 524, number of deaths = 508 concordance = 0.60, Wald test = 46.77). Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded.

	coef	exp(coef)	se(coef)	z	Pr (>|z|)
Injury treatment	0.16	1.17	0.21	0.77	0.44
Infection treatment	**0.77**	**2.16**	**0.22**	**3.52**	**<0.001**
Protein	0.05	1.06	0.08	0.69	0.49
Protein^2	**0.57**	**1.78**	**0.13**	**4.55**	**<0.001**
Injury:Protein	-0.10	0.91	0.11	-0.88	0.38
Infection:Protein	0.16	1.18	0.13	1.22	0.22
Injury:Protein^2	-0.17	0.84	0.18	-0.99	0.32
Infection:Protein^2	**-0.57**	**0.57**	**0.20**	**-2.86**	**0.004**

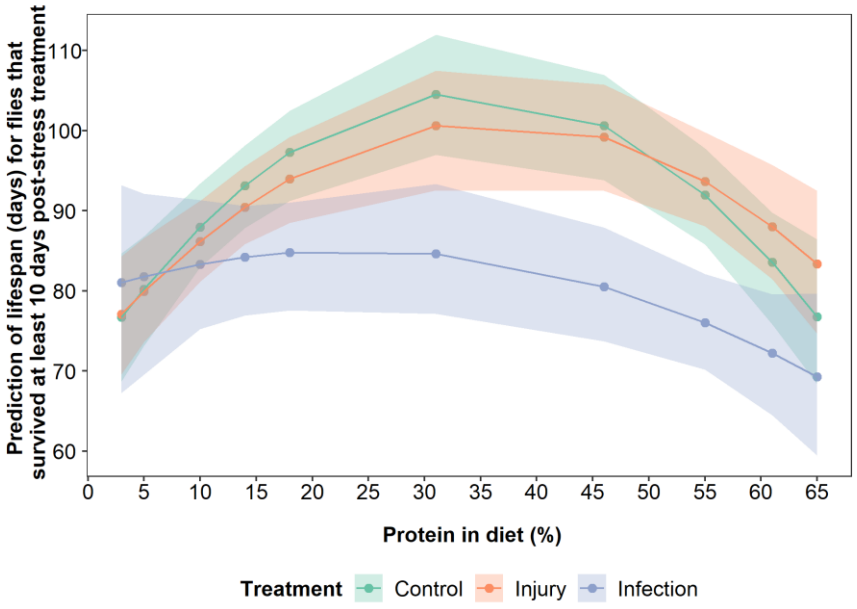

Figure S2.8: Model predictions of the effects of protein restriction on lifespan of flies infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines). Shaded areas are 95% 95% credible intervals. Protein and protein2 are mean centered to standard deviation of 1.

Table S2.6: Model summary of effects of protein restriction and stress treatments on lifespan with flies that survived at least 10 days post-stress treatment. Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded.

	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
Intercept	**105.00**	**97.80**	**112.47**	**1000**	**<0.001**
Injury treatment	-3.94	-13.01	8.03	1000	0.44
Infection treatment	**-20.68**	**-31.16**	**-8.25**	**1000**	**<0.001**
Protein	1.82	-2.43	5.88	1000	0.40
Protein^2	**-15.03**	**-21.31**	**-8.96**	**1000**	**<0.001**
Injury:Protein	1.66	-4.13	7.03	1000	0.57
Infection:Protein	-6.01	-13.14	0.34	1000	0.07
Injury:Protein^2	3.94	-4.78	12.33	1000	0.35
Infection:Protein^2	**10.33**	**0.93**	**20.71**	**1000**	**0.04**

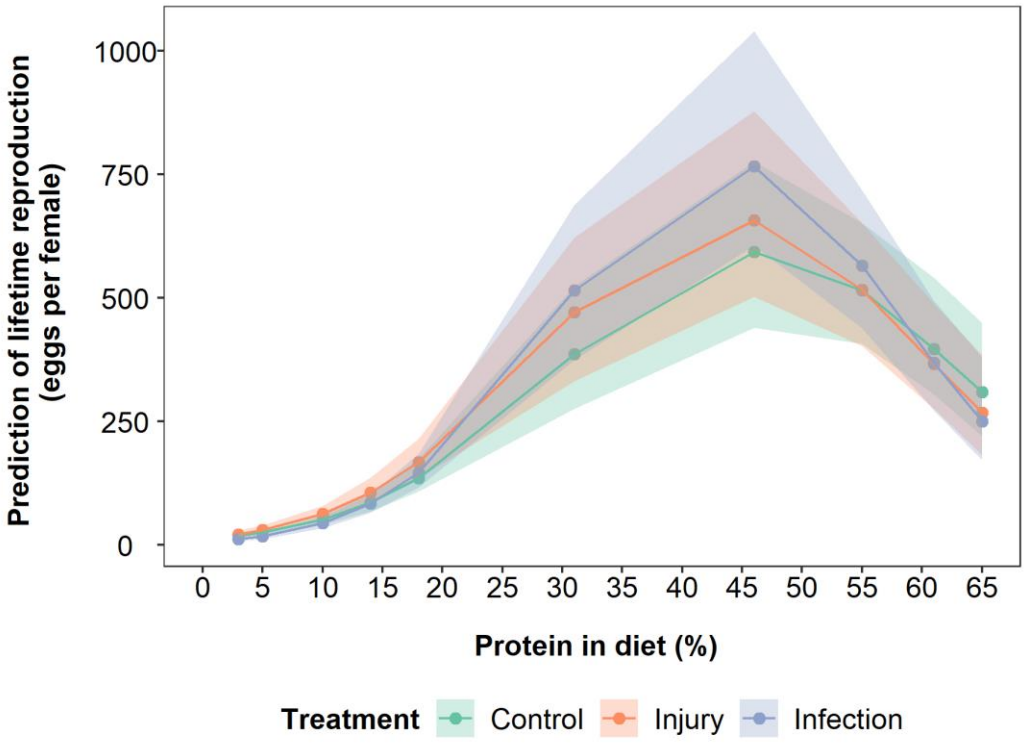

Figure S2.9: Model predictions of the effects of protein restriction on the lifetime number of eggs produced by flies infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines), when accounting for lifespan (mean centred). Shaded areas are 95% credible intervals. Protein and protein^2 are mean centered to standard deviation of 1.

Table S2.7: Model summary of effects of protein restriction and stress treatments on lifetime eggs produced. Mean centered lifespan is added as a fixed effect to remove the effect of lifespan on reproduction. Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level α = 0.05 are bolded.

	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
Intercept	**5.94**	**5.64**	**6.28**	**1000**	**<0.001**
Injury treatment	0.20	-0.19	0.67	1000	0.36
Infection treatment	0.29	-0.18	0.72	1000	0.21
Protein	**1.31**	**1.15**	**1.50**	**1119**	**<0.001**
Protein^2	**-0.97**	**-1.24**	**-0.70**	**1000**	**<0.001**
Lifespan	**0.93**	**0.83**	**1.04**	**1000**	**<0.001**
Injury:Protein	-0.81	-0.33	0.17	1000	0.52
Infection:Protein	0.18	-0.07	0.45	1000	0.17
Injury:Protein^2	-0.10	-0.45	0.28	1000	0.60
Infection:Protein^2	-0.34	-0.71	0.05	1000	0.08

7.3 Appendix C: Chapter 3 supplement

7.3.1 Supplementary methods:

7.3.1 Bacterial growth (CFU) measurements:

24 hours post-infection two replicate groups of three flies from the infected, sham and control groups were plated (following Gupta et al., 2017). Across infection blocks, colonies grew on the plates confirming successful infections, except for the first block where initially only one fly per sample was used for the plating. Infected flies from the first block showed similar levels of mortality to flies from other blocks, suggesting they were indeed infected and that use of only a single fly resulted in bacterial levels that were below a detection threshold in the assay. Due to logistical reasons, the last block of infections was plated 48 hours post-infection, however another group of infected flies from the same overnight bacterial culture showed growth (Halonen, data not shown).

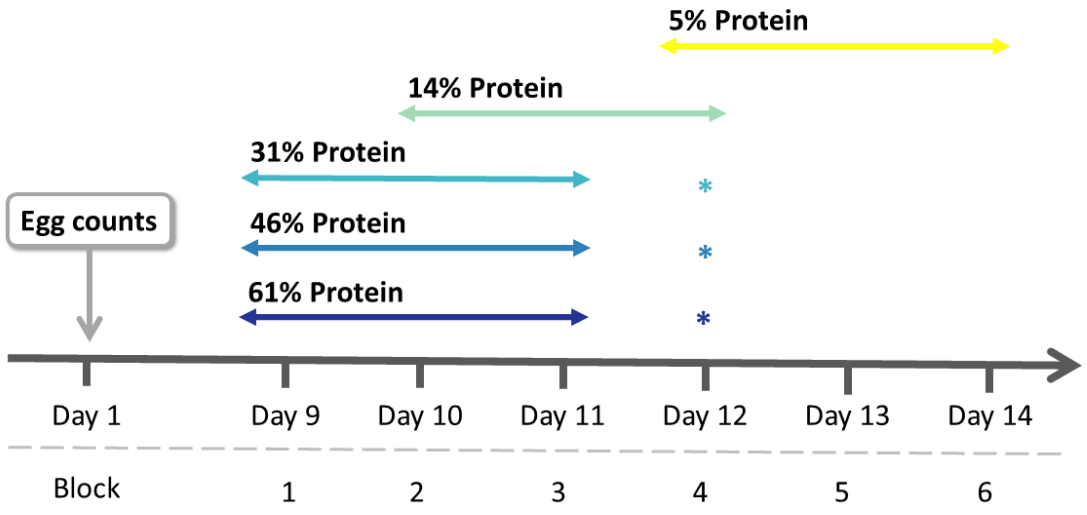

Figure S3.1: Schematic for adult collection across days to create 6 blocks of females. Stars (*) indicate if only a few addition adults were collected on this day to reach sample size per diet (see methods).

Table S3.1: Total sample size per diet and treatment of flies collected across three to four days after eclosion started.

Protein in diet (%)	P:C ratio	Stress treatment		
		Control	Injury	Infection
5	1:16	35	40	35
14	1:6	32	36	30
31	1:2	25	24	25
46	1:1	19	19	18
61	2:1	22	24	23

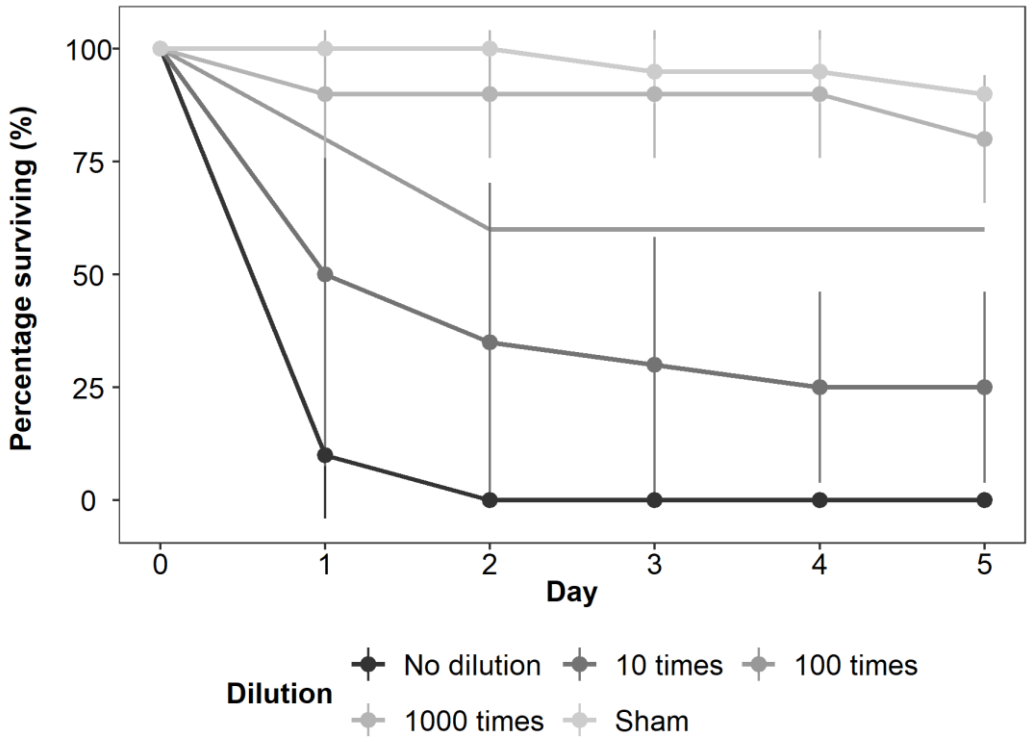

Figure S3.2: Dilution series for *Pseudomonas entomophila* bacterial solution from the same stock as used in infections. 10 females per vial were infected with the specified solution (no dilution to 1000 times dilution) or with no pathogen ("Sham"). Results show mean survival of two replicates of ten flies and the vertical lines indicate standard deviation, except for the 100 times dilution, which only has one replicate.

7.3.2 Supplementary results:

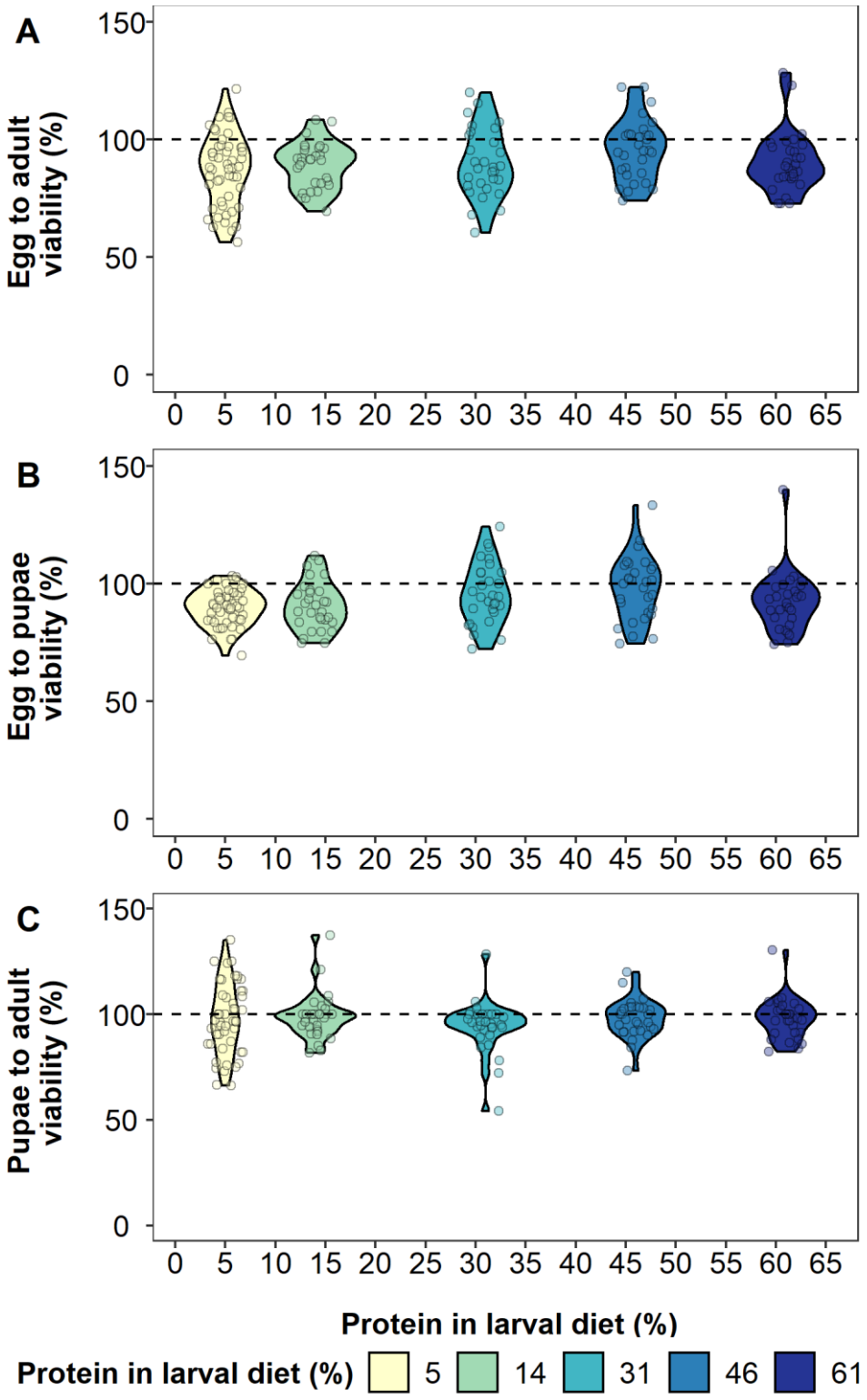

Figure S3.3: Effects of protein in larval diet on the percentage of eggs developing to adults (A), eggs developing to pupae (B) and pupae developing to adults (C). Values are over 100% due to inaccuracies in egg and pupal counts.

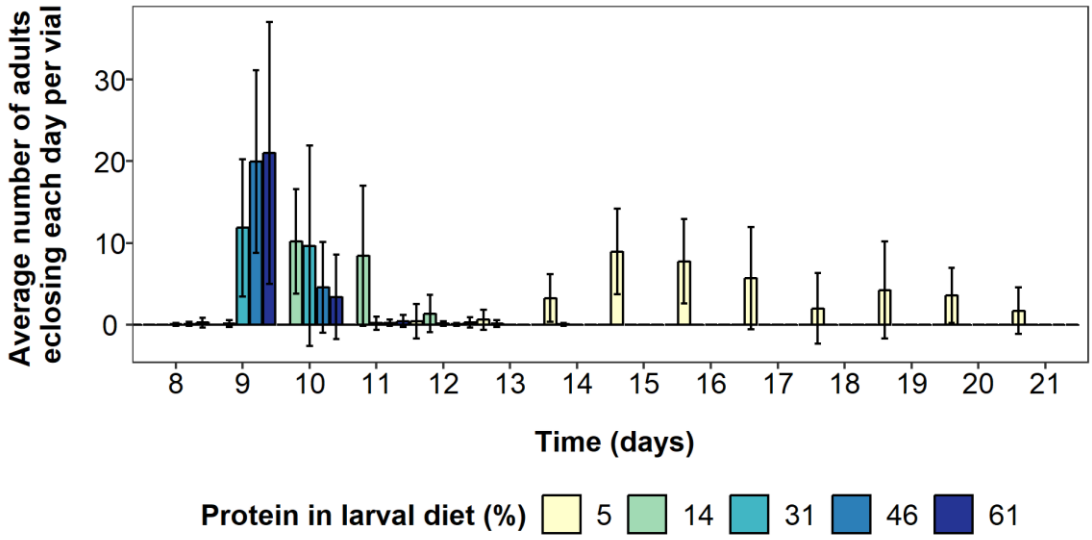

Figure S3.4: Effects of protein in larval diet on the average number of adult flies eclosing each day after egg production. No adults eclosed prior to day 8, so these days are not shown. Error bars are standard deviations.

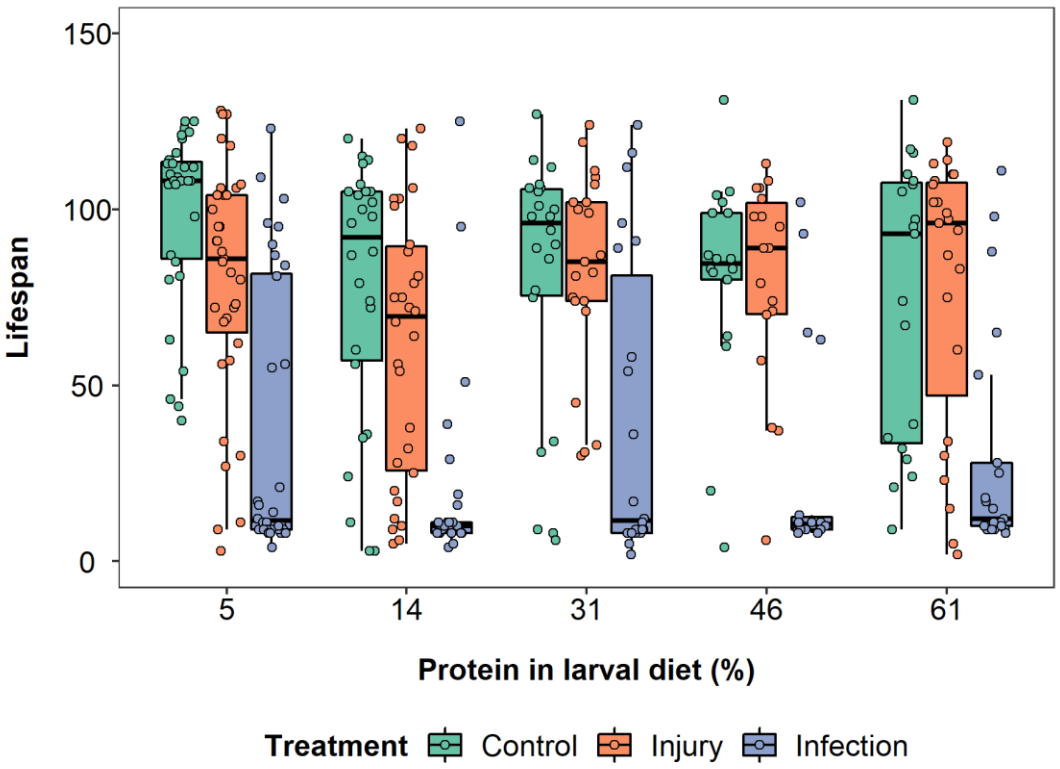

Figure S3.5: Effects of protein in larval diet on the lifespan of flies infected with a bacterial pathogen (blue bars and data points), injured by a pinprick (orange bars and data points) or with no treatment (green bars and data points). The lines in the box plots indicates median values (50% quantile), boxes are the interquartile range (25% to 75% quantiles) and whiskers are minimum or maximum quartiles (25% - 1.5 x interquartile range, 75% + 1.5 x interquartile range).

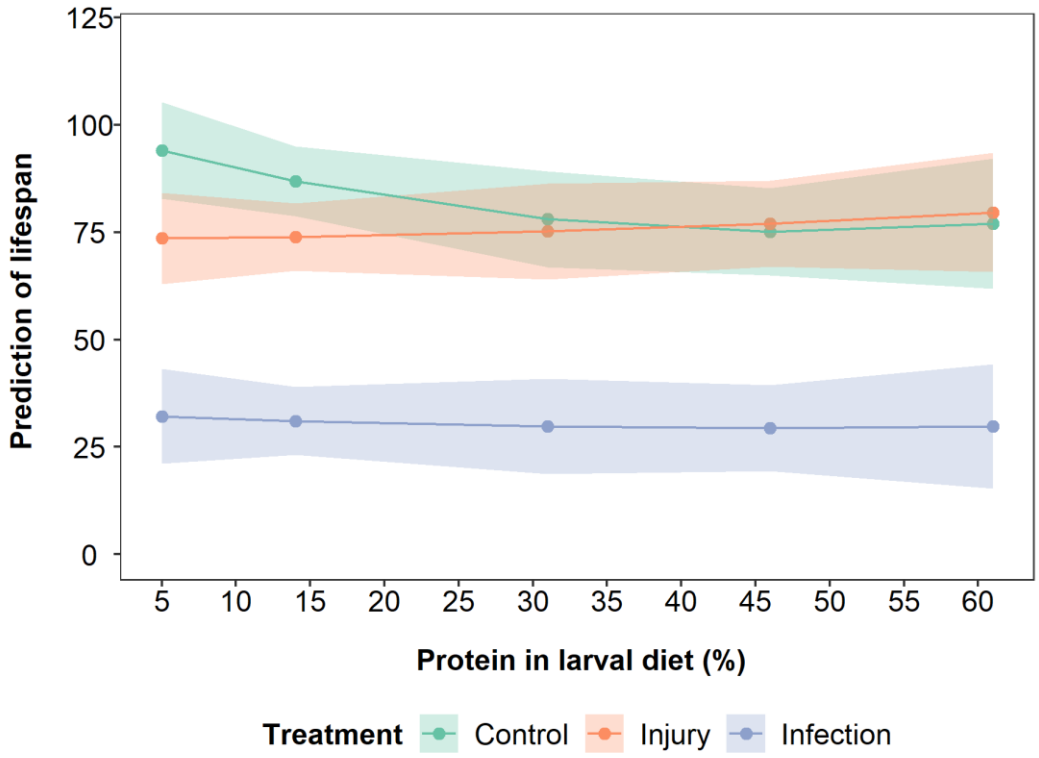

Figure S3.6: Model predictions of the effects of larval protein restriction on adult lifespan of flies infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines). Shaded areas are 95% confidence intervals.

Table S3.2: Summary of a linear model analysing effects of P:C in larval diet and stress treatments on lifespan of adult flies with (A) main effects parameter estimates and associated LRT tests and (B) for the full model. Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	Z value	Df	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:						
Injury treatment	**-8.30**	**4.51**	**-1.84**	**2**	**134.08**	**<0.001**
Infection treatment	**-53.08**	**4.56**	**-11.65**			
Protein	-1.58	1.85	-0.85	1	0.74	0.39
Protein^2	1.89	2.56	0.73	1	0.60	0.44
(B) Full model parameter estimates and LRT test values for interactions:						
Intercept	79.36	5.51	14.40			
Injury treatment	-4.53	7.65	-0.59			
Infection treatment	-49.44	7.62	-6.49			
Protein	-8.67	3.86	-2.25			
Protein^2	4.42	4.47	0.99			
Injury:Protein	10.54	5.26	2.01	2	4.24	0.12
Infection:Protein	7.41	5.32	1.39			
Injury:Protein^2	-3.76	6.18	-0.61	2	0.49	0.78
Infection:Protein^2	-3.71	6.23	-0.60			

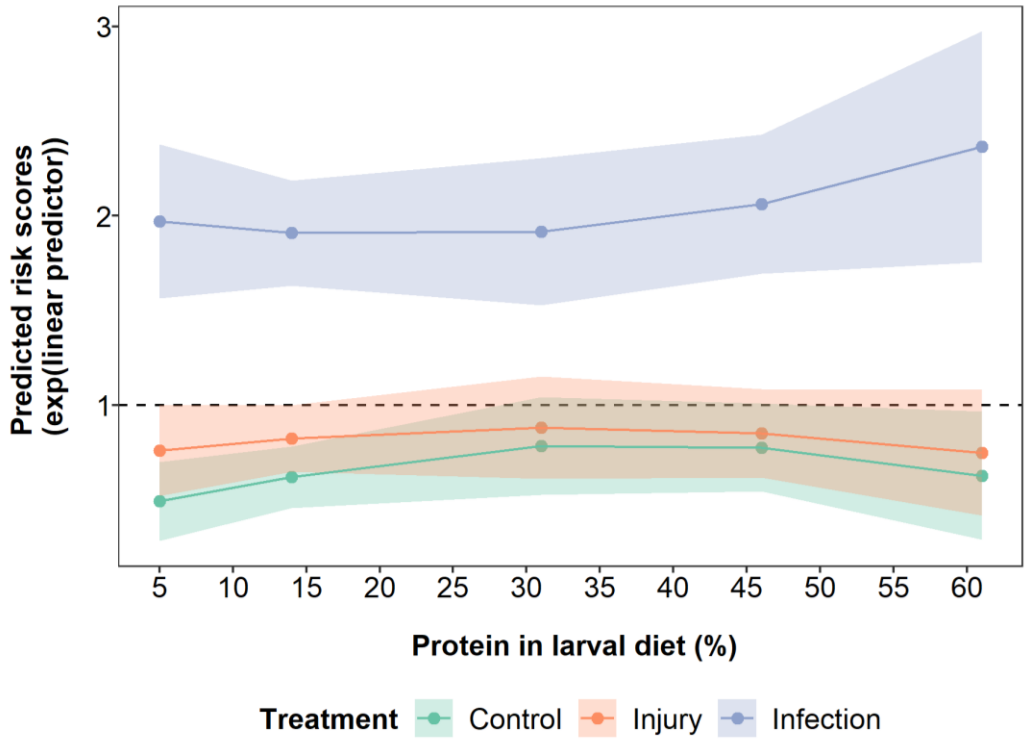

Figure S3.7: Model predictions of the effects of larval protein restriction on survival of flies infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines). $y = 1$ line shows no change in risk ratio, i.e. treatment would have no effect compared to baseline hazard. Shaded areas are 95% confidence intervals.

Table S3.3: Model summary of a Cox Proportional Hazard regression model of the effects of protein in larval diet and stress treatments on survival (n = 407, number of deaths = 365, concordance = 0.672, R^2 = 0.18). Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level α = 0.05 are bolded.

	coef	exp(coef)	se(coef)	z	Pr (>|z|)
Injury treatment	1.15	1.15	021	0.67	0.50
Infection treatment	**0.92**	**2.50**	**0.21**	**4.32**	**<0.001**
Protein	0.20	1.22	0.11	1.82	0.07
Protein^2	-0.19	0.83	0.12	-1.54	0.12
Injury:Protein	-0.16	0.86	0.15	-1.03	0.30
Infection:Protein	-0.17	0.84	0.15	-1.11	0.27
Injury:Protein^2	0.11	1.11	0.17	0.63	0.53
Infection:Protein^2	0.25	1.29	0.17	1.48	0.14

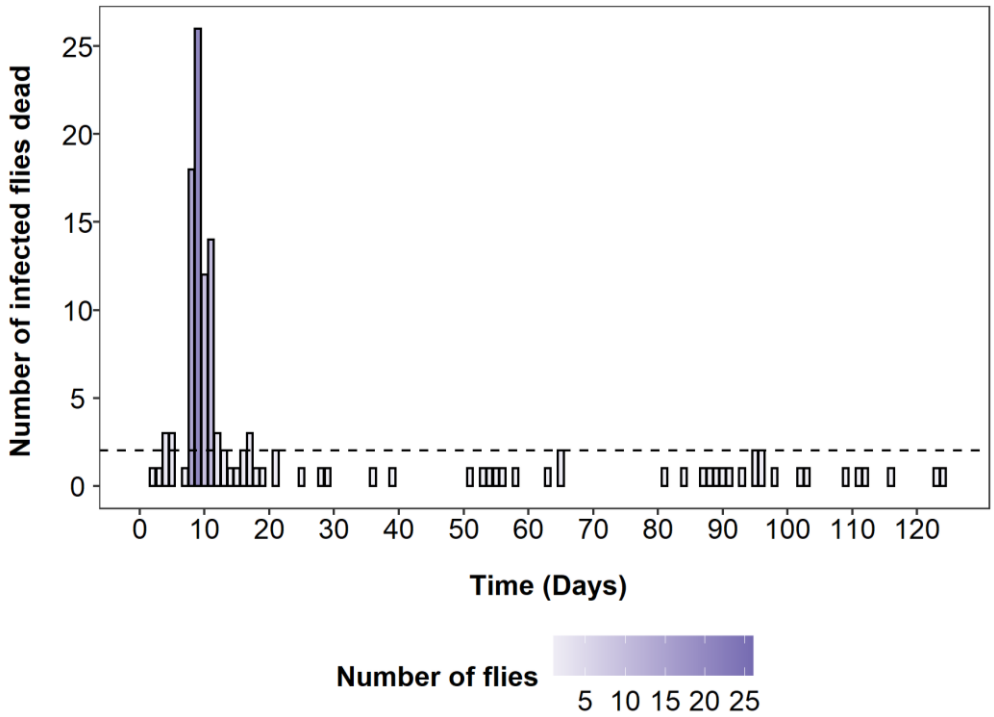

Figure S3.8: Number of flies dead post-infection treatment. The dotted line shows where the number of dead flies per day is 2.

Table S3.4: Sample sizes for flies when separated into early-mortality post-stress treatment (dying or going missing prior to day 13) and later-life mortality (dying or going missing after 13 days).

Stress treatment	Diet (protein %)	Number of flies dead or missing prior to day 13	Number of flies dead or missing after day 13
Control	5	3	32
	14	5	27
	31	6	19
	46	2	17
	61	3	19
Injury	5	7	33
	14	11	25
	31	2	22
	46	2	17
	61	2	22
Infection	5	20	15
	14	23	7
	31	14	11
	46	14	4
	61	13	10

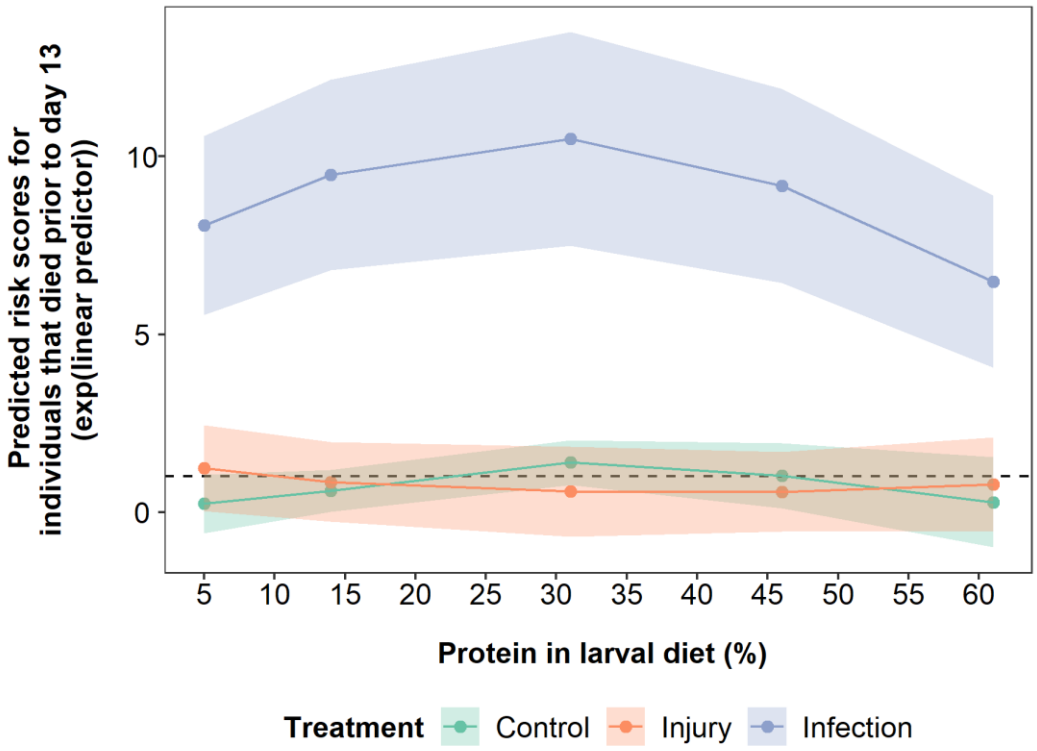

Figure S3.9: Model predictions of the effects of larval protein restriction on survival of flies that did not survive until 13 days post-stress treatments, and were infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines). $y = 1$ line shows no change in risk ratio, i.e. treatment would have no effect compared to baseline hazard. Shaded areas are 95% confidence intervals.

Table S3.5: Model summary of a Cox Proportional Hazard regression model of the effects of protein in larval diet and stress treatments on survival of flies that did not survive until 13 days post-stress treatments (n = 407, number of deaths = 95, concordance = 0.8). Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded.

	coef	exp(coef)	se(coef)	z	Pr (>|z|)
Injury treatment	-0.75	0.47	0.76	-0.99	0.32
Infection treatment	**2.10**	**8.18**	**0.50**	**4.16**	**<0.001**
Protein	0.60	1.83	0.51	1.18	0.24
Protein^2	-0.92	0.40	0.55	-1.69	0.09
Injury:Protein	-0.95	0.39	0.62	-1.52	0.13
Infection:Protein	-0.57	0.57	0.53	-1.07	0.28
Injury:Protein^2	1.22	3.39	0.70	1.75	0.08
Infection:Protein^2	0.73	2.07	0.57	1.28	0.20

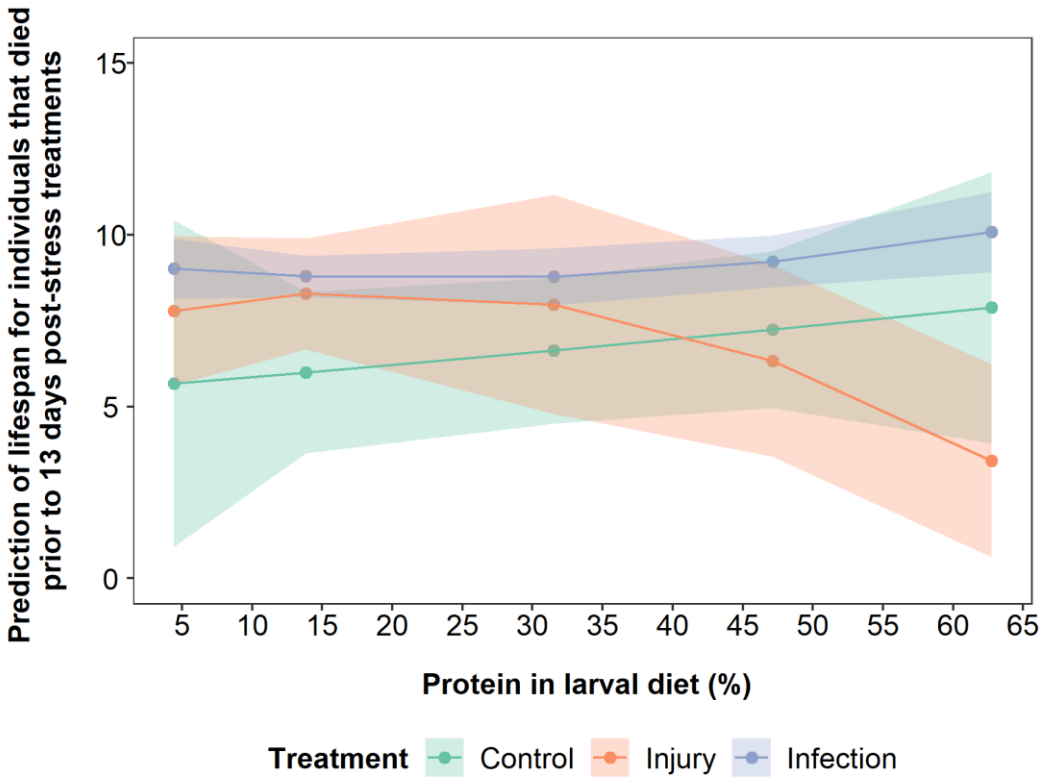

Figure S3.10: Model predictions of the effects of larval protein restriction on adult lifespan of flies that died prior to 13 days post-stress treatments, where flies were infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines). Shaded areas are 95% confidence intervals.

Table S3.6: Summary of a linear model analysing effects of P:C in larval diet and stress treatments on lifespan of adult flies that died prior to 13 days post-stress treatments with (A) main effects parameter estimates and associated LRT tests and (B) for the full model. Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	Z value	Df	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:						
Injury treatment	**0.32**	**1.03**	**0.31**	**2**	**15.13**	**<0.001**
Infection treatment	**2.43**	**0.82**	**2.98**			
Protein	0.11	0.24	0.46	1	0.25	0.61
Protein^2	0.26	0.31	0.85	1	0.63	0.43
(B) Full model parameter estimates and LRT test values for interactions:						
Intercept	6.48	0.99	6.55			
Injury treatment	1.71	1.81	0.94			
Infection treatment	2.26	1.06	2.12			
Protein	0.76	1.37	0.56			
Protein^2	0.03	1.21	0.03			
Injury:Protein	-1.65	1.65	-1.00	2	1.57	0.46
Infection:Protein	-0.60	1.40	-0.43			
Injury:Protein^2	-1.15	1.61	-0.71	2	2.00	0.37
Infection:Protein^2	0.34	1.25	0.27			

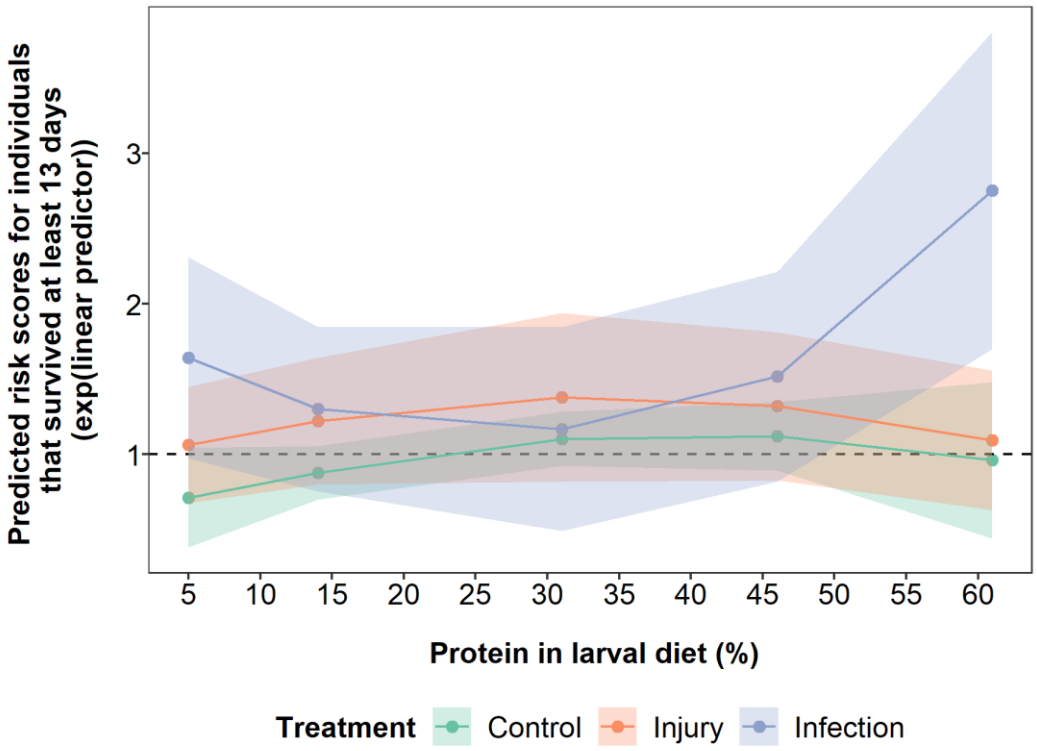

Figure S3.11: Model predictions of the effects of larval protein restriction on survival of flies that survived at least 13 days post-eclosion and were infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines). $y = 1$ line shows no change in risk ratio, i.e. treatment would have no effect compared to baseline hazard. Shaded areas are 95% confidence intervals.

Table S3.7: Model summary of a Cox Proportional Hazard regression model of the effects of protein in larval diet and stress treatments on survival (n = 280, number of deaths = 270, concordance = 0.59). Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded.

	coef	exp(coef)	se(coef)	z	Pr (>|z|)
Injury treatment	0.24	1.28	0.22	1.08	0.28
Infection treatment	0.07	1.08	0.30	0.24	0.81
Protein	0.20	1.22	0.11	1.77	0.08
Protein^2	-0.17	0.85	0.13	-1.25	0.21
Injury:Protein	-0.11	0.89	0.16	-0.73	0.47
Infection:Protein	-0.18	0.84	0.20	-0.88	0.38
Injury:Protein^2	0.03	1.03	0.18	0.45	0.88
Infection:Protein^2	**0.49**	**1.63**	**0.24**	**2.10**	**0.04**

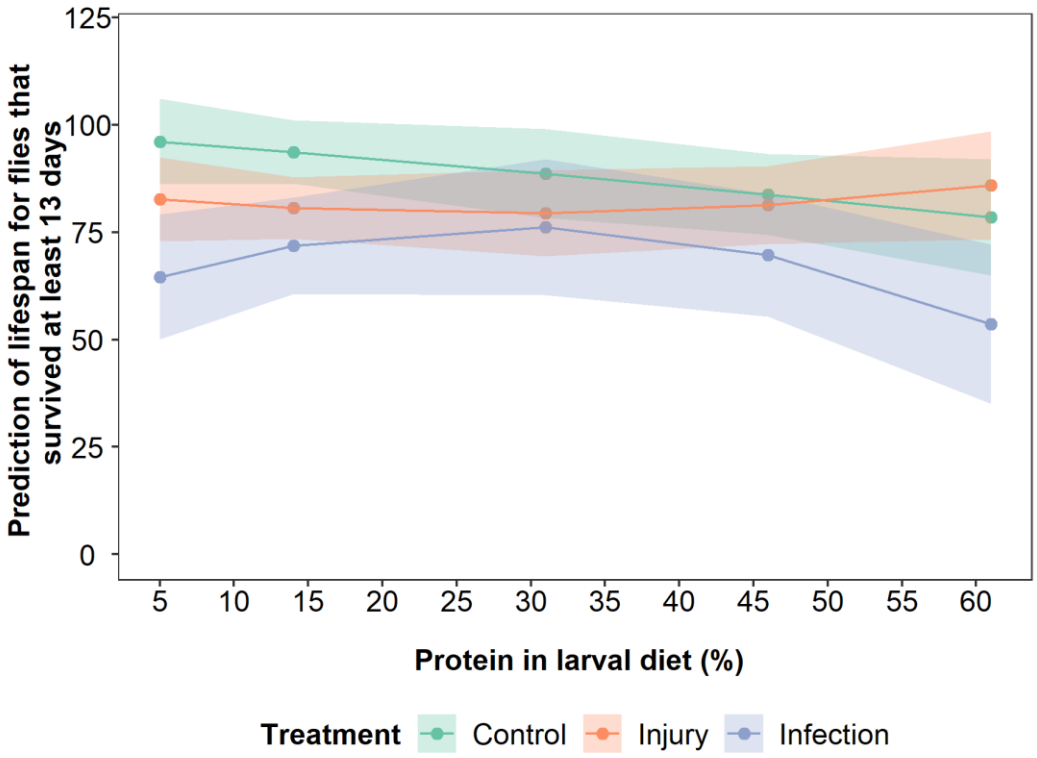

Figure S3.12: Model predictions of the effects of larval protein restriction on adult lifespan of flies that survived at least 13 days post-stress treatments, where flies were infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines). Shaded areas are 95% confidence intervals.

Table S3.8: Summary of a linear model analysing effects of P:C in larval diet and stress treatments on lifespan of adult flies that survived at least 13 days post-stress treatments with (A) main effects parameter estimates and associated LRT tests and (B) for the full model. Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	Z value	Df	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:						
Injury treatment	**-7.34**	**4.04**	**-1.82**	**2**	**18.34**	**<0.001**
Infection treatment	**-22.97**	**5.32**	**-4.32**			
Protein	-2.56	1.84	-1.39	1	1.97	0.16
Protein^2	-0.65	2.58	-0.25	1	0.05	0.82
(B) Full model parameter estimates and LRT test values for interactions:						
Intercept	89.71	5.10	17.59			
Injury treatment	-10.32	6.85	-1.51			
Infection treatment	-13.42	9.14	-1.47			
Protein	-6.36	3.47	-1.83			
Protein^2	-0.40	4.43	-0.10			
Injury:Protein	6.22	4.66	1.33	2	2.29	0.32
Infection:Protein	7.09	6.05	1.17			
Injury:Protein^2	3.06	5.65	0.54	2	2.84	0.24
Infection:Protein^2	-8.98	7.18	-1.25			

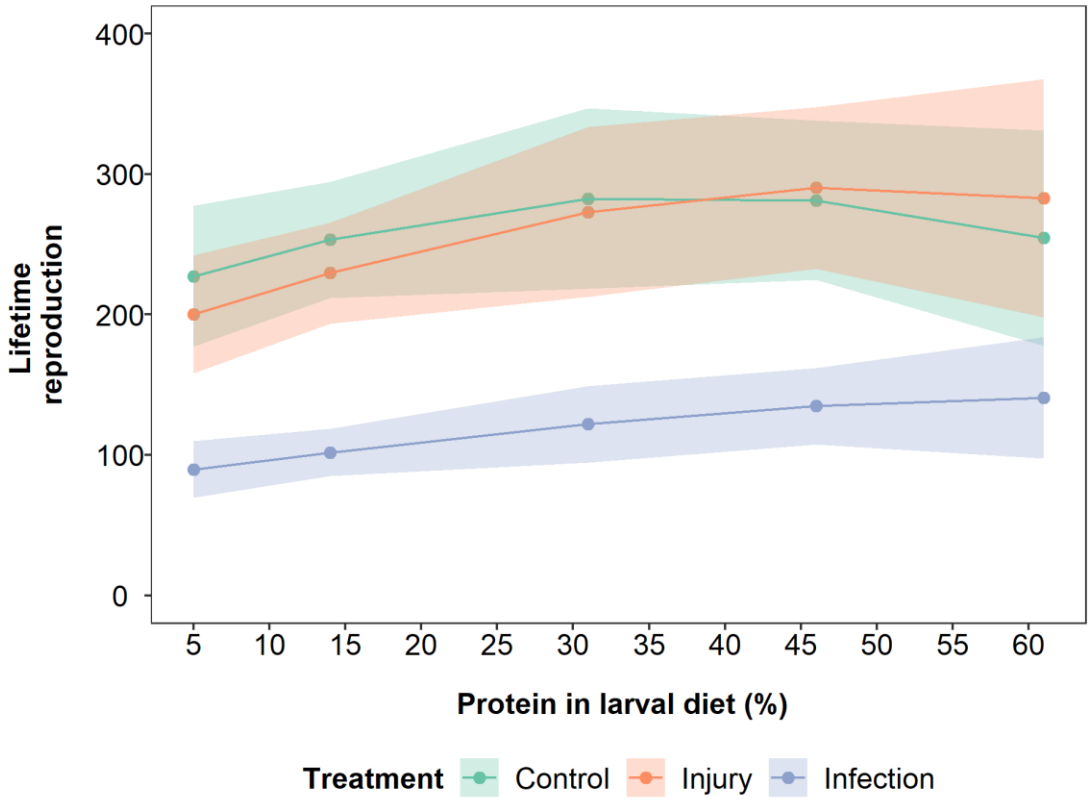

Figure S3.13: Model predictions of the effects of larval protein restriction on lifetime egg production (up to day 98) of flies infected with a bacterial pathogen (blue data points and lines), injured by a pinprick (orange data points and lines) or with no treatment (green data points and lines). Shaded areas are 95% confidence intervals.

Table S3.9: Summary of a zero-inflated negative binomial model analysing effects of P:C in larval diet and stress treatments on the total number of eggs produced per fly with (A) main effects parameter estimates and associated LRT tests and (B) for the full model. Protein and protein^2 are mean centered to standard deviation of 1. Significant results below significance level $\alpha = 0.05$ are bolded. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	Z value	Df	Chisq	Pr (>Chisq)
(A) Main effects parameter estimates and LRT test values:						
Injury treatment	**-0.04**	**0.09**	**-0.48**	**2**	**80.68**	**<0.001**
Infection treatment	**-0.82**	**0.09**	**-8.82**			
Protein	**0.12**	**0.04**	**3.15**	**1**	**5.91**	**0.02**
Protein^2	-0.07	0.05	-1.45	1	2.10	0.15
(B) Full model parameter estimates and LRT test values for interactions:						
Intercept	5.67	0.11	50.99			
Injury treatment	-0.05	0.16	-0.32			
Infection treatment	-0.86	0.16	-5.54			
Protein	0.09	0.08	1.21			
Protein^2	-0.09	0.09	-0.99			
Injury:Protein	0.08	0.11	0.75	2	0.98	0.61
Infection:Protein	0.10	0.11	0.93			
Injury:Protein^2	0.008	0.13	0.07	2	0.10	0.95
Infection:Protein^2	0.04	0.13	0.29			

7.4 Appendix D: Chapter 4 supplement

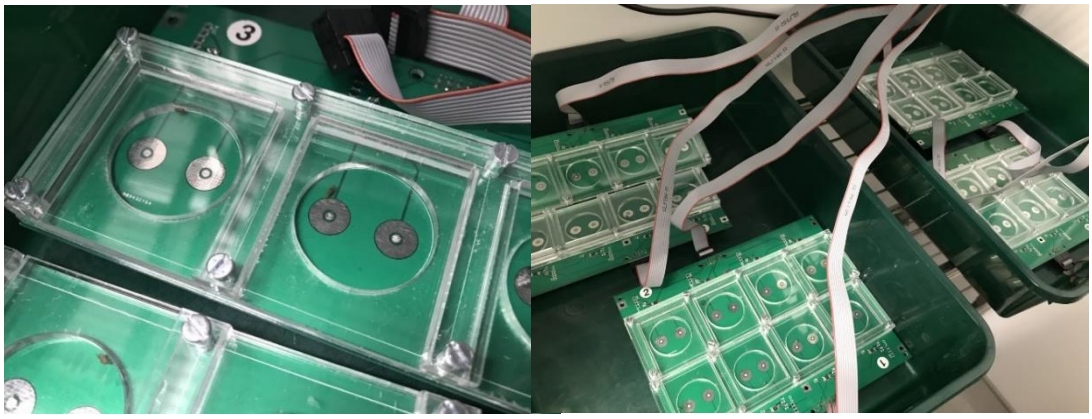

Figure S4.1: FlyPAD (Itskov et al., 2014) experimental set up shown as the individual arenas with experimental flies (left image) and a full experimental set up with 30 out of 32 arenas used (right image). In this run through, food on the left electrode is 0:1 P:C and food on the right electrode is 1:4 P:C (Image by Eevi Savola).

7.4.1 Supplementary statistical methods:

To confirm effects seen in parametric models, various non-parametric tests were completed. For preference index, to confirm the effect of treatment, a Kruskal-Wallis test was used. As a value of 0 on the preference index scale indicates no preference, we ran Wilcoxon rank sum tests to see whether preference index was statistically different from 0. This was done for the whole dataset and for each treatment specifically. For infected flies, the effect of presence of quantifiable bacterial growth on preference index was analysed with Kruskal-Wallis test.

Wilcoxon rank sum tests confirmed that preference index did not differ from 0 either overall ($V = 6159$, $p = 0.84$) or in each treatment separately (Control: $V = 628$, $p = 0.68$; Injury: $V = 770$, $p = 0.46$; Infection: $V = 690$, $p = 0.50$). A Kruskal-Wallis test confirmed that treatment had no effect on preference index ($\chi^2 = 1.40$, $df = 2$, $p = 0.50$). A Kruskal-Wallis test confirmed that the presence of bacterial growth had no effect on preference index in infected flies ($\chi^2 = 0.58$, $df = 1$, $p = 0.45$).

7.4.2 Supplementary results:

Table S4.1: Summary of a zero-inflated negative binomial model of the effects of treatment and total sips taken on the number of 1:4 P:C sips taken with (A) main effects parameter estimates and associated LRT tests and (B) for the full model. Significant results below significance level α = 0.05 are bolded. (A) The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect.

	Estimate	Standard error	T value	Df	Chisq	Pr (>Chisq)
Intercept	4.75	0.21	23.04			
Total sips	**1.64**	**0.20**	**8.25**	**1**	**85.13**	**<0.001**
Injury treatment	-0.08	0.27	-0.28	2	1.06	0.59
Infection treatment	-0.27	0.27	-1.00			
Intercept	4.80	0.22	22.24			
Total sips	1.31	0.30	4.42			
Injury treatment	-0.12	0.28	-0.44			
Infection treatment	-0.33	0.28	-1.18			
Total sips:Injury treatment	0.29	0.41	0.71	2	2.68	0.26
Total sips:Infection treatment	0.72	0.44	1.63			

7.5 Appendix E: Chapter 5 supplement

7.5.1 Supplementary methods:

Table S5.1: Number of flies injured and infected per each diet combination for each collection time point.

Infection time block	P:C ratio	Protein in diet (%)	Treatment	
			Injury	Infection
16	1:16	5	45	69
	1:1	46	46	68
24	1:16	5	47	64
	1:1	46	47	63
48	1:16	5	45	58
	1:1	46	44	58
Total	1:16	5	137	191
	1:1	46	137	189

Table S5.2: Total sample size per diet and treatment of flies in survival analysis.

Collection time point	P:C ratio	Protein in diet (%)	Treatment	
			Injury	Infection
16	1:16	5	11	34
	1:1	46	12	34
24	1:16	5	10	31
	1:1	46	12	29
48	1:16	5	10	24
	1:1	46	11	24
Total	1:16	5	31	89
	1:1	46	35	87

Table S5.3: Information on each AMP and reference gene, including primer sequence and source, optimisation temperature used in qPCR in the annealing step and efficiency at this temperature.

AMP	F/R	Primer sequence	Primer from previous study	Optimisation temperature (°C) for annealing step	Efficiency at optimisation temperature (%)
Rpl32	F	ATGCTAAGCTGTCGCACAAATG	Ponton et al., 2011	60	92.94
	R	GTTCGATCCGTAACCGATGT			
Attacin C (AttC)	F	TGCCCGATTGGACCTAAGC	Troha et al., 2018	62	97
	R	GCGTATGGGTTTTGGTCAGTTC			
Cecropin A1 (CecA1)	F	GGACAATCGGAAGCTGGTT	Bastos et al., 2017	55	112
	R	TGTGCTGACCAACACGTTC			
Diptericin (Dpt)	F	GACGCCACGAGATTGGACTG	Lee et al., 2017	60	111.19
	R	CCCACTTTCCAGCTCGGTTC			
Drosocin (Dro)	F	TGTCCACCACTCCAAGCACAA	Lee et al., 2017	62	101
	R	CATGGCAAAAACGCAAGCAA			
Drosomycin (Drs)	F	CGTGAGAACCTTTTCCAATATGATG	Troha et al., 2018	60	109.71
	R	TCCCAGGACCACCAGCAT			

Table S5.4: Total sample size of samples (groups of three flies) per diet and treatment of flies used in the AMP analysis.

AMP	P:C ratio	P:C ratio	Time post-infection (h)	Treatment	
				Injury	Infection
Attacin C	1:16	1:16	16	6	7
			24	7	6
			48	8	8
	1:1	1:1	16	7	8
			24	6	8
			48	8	8
Cecropin A1	1:16	1:16	16	6	6
			24	8	7
			48	6	7
	1:1	1:1	16	5	8
			24	6	8
			48	8	8
Diptericin	1:16	1:16	16	7	8
			24	8	7
			48	7	8
	1:1	1:1	16	8	8
			24	7	7
			48	8	8
Drosocin	1:16	1:16	16	8	8
			24	6	7
			48	8	8
	1:1	1:1	16	8	8
			24	7	8
			48	8	8

Drosomycin	1:16	1:16	16	8	7
			24	8	7
			48	8	8
	1:1	1:1	16	8	8
			24	7	8
			48	8	8

7.5.2 Supplementary results:

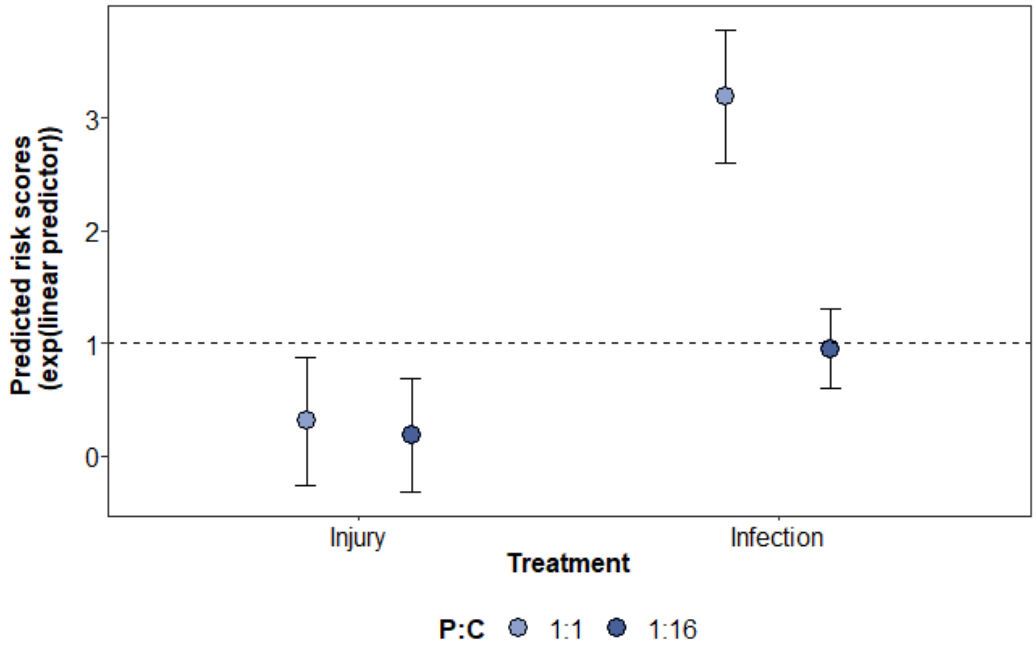

Figure S5.1: Cox proportional hazards model predictions for the effects of P:C on survival post-infection treatment for only (A) injured or (B) infected individuals. y = 1 line shows no change in risk ratio, i.e. treatment would have no effect compared to baseline hazard. Lighter purple points show model predictions for the lower P:C diet (1:16 P:C) and darker purple points show model predictions for the higher P:C diet (1:1 P:C). Error bars show 95% confidence intervals.

Table S5.5: Summary of Cox proportional hazards regression model analysing effects of diet and infection treatment on survival. $N = 241$ with 86 deaths. Significant results below significance level α = 0.05 are bolded.

	Coef	Exp(coef)	Se(coef)	Z	Pr (>|z|)
46 % protein	-0.57	0.56	0.91	-0.62	0.53
Infection treatment	**2.33**	**10.32**	**0.59**	**3.93**	**<0.001**
1:1 P:C:Infection treatment	-0.64	0.53	0.94	-0.68	0.50

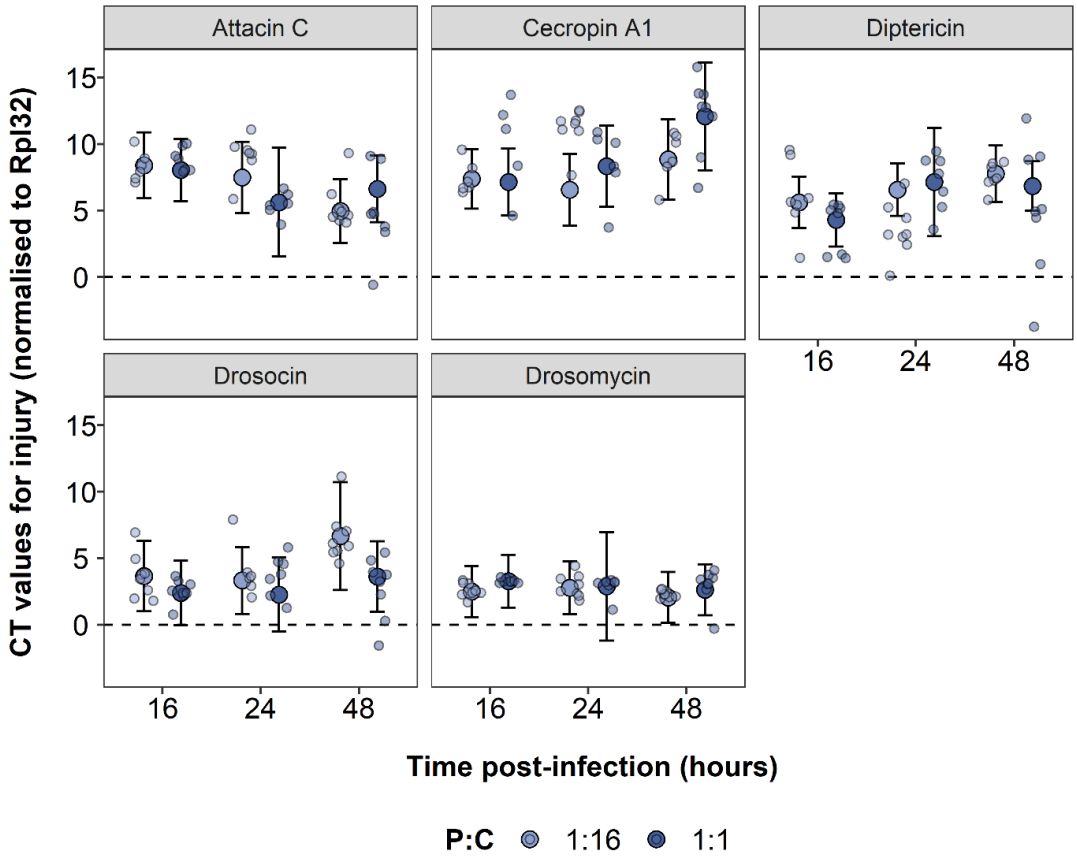

Figure S5.2: Effects of dietary P:C and time-post infection on AMP gene expression of injured flies normalised to the Rpl32 gene. Lighter purple points show data for the lower P:C diet (1:16 P:C) and darker purple points show data for the higher P:C diet (1:1 P:C). Linear model predictions are shown as additional data points with associated error bars showing 95% confidence intervals. Dotted line shows y = 0.

Table S5.6: Summary of main effects parameter estimates and associated LRT test values for a linear mixed effects model analysing effects of diet, time after infection and AMP on the mean C_T value for injury treatment. The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect. Sample and qPCR plate are included as random effects. Significant results below significance level $\alpha = 0.05$ are bolded.

	Estimate	Standard error	T value	Chisq	Pr (>Chisq)
1:1 P:C	-0.16	0.46	-0.34	0.11	0.74
24 hours post injury	-0.09	0.60	-0.15	0.75	0.69
48 hours post injury	0.41	0.63	0.66		
Cecropin A1	**2.24**	**0.99**	**2.25**	**39.90**	**<0.001**
Diptericin	**0.08**	**0.89**	**0.09**		
Drosocin	**-2.69**	**1.07**	**-2.52**		
Drosomycin	**-3.61**	**0.89**	**-4.03**		

Table S5.7: Summary of two-way interaction estimates and associated LRT test values for a linear mixed effects model analysing effects of diet, time after infection and AMP on the mean C_T value for injury treatment. Estimates and standard errors are from a model not including the three-way interaction. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect. Sample and qPCR plate are included as random effects. Significant results below significance level $\alpha = 0.05$ are bolded.

	Estimate	Standard error	T value	Chisq	Pr (>Chisq)
Intercept	7.60	1.04	7.29		
1:1 P:C	-0.29	0.89	-0.32		
24 hours post-injury	-0.90	1.15	-0.78		
48 hours post-injury	-1.95	1.147	-1.72		
Cecropin A1	0.05	1.29	0.04		
Diptericin	-1.93	1.12	-1.72		
Drosocin	-3.51	1.35	-2.60		
Drosomycin	-4.98	1.12	-4.43		
1:1 P:C:24 hours post injury	0.57	1.32	0.43	0.17	0.92
1:1 P:C:48 hours post injury	0.28	1.09	0.26		
1:1 P:C:Cecropin A1	**0.80**	**0.79**	**1.00**	**15.76**	**0.003**
1:1 P:C:Diptericin	**-0.93**	**0.79**	**1.00**		
1:1 P:C:Drosocin	**-1.39**	**0.96**	**-1.45**		
1:1 P:C:Drosomycin	**0.76**	**0.68**	**1.13**		
24 hours post injury:Cecropin A1	**0.93**	**1.16**	**0.81**	**20.85**	**0.008**
48 hours post injury:Cecropin A1	**3.75**	**1.51**	**2.49**		
24 hours post injury:Diptericin	**1.96**	**1.07**	**1.84**		
48 hours post injury:Diptericin	**4.12**	**1.13**	**3.65**		

24 hours post injury:Drosocin	**0.17**	**1.31**	**0.13**		
48 hours post injury:Drosocin	**3.33**	**1.34**	**2.49**		
24 hours post injury:Drosomycin	**0.96**	**1.12**	**0.86**		
48 hours post injury:Drosomycin	**1.32**	**1.12**	**1.19**		

Table S5.8: Summary of a linear mixed effects model analysing effects of model analysing effects of diet, time after infection and AMP on the mean C_T value for injury treatment. Sample and qPCR plate are included as random effects.

	Estimate	Standard error	T value	Chisq	Pr (>Chisq)
Intercept	8.39	1.27	6.62		
1:1 P:C	-0.34	0.95	-0.36		
24 hours post injury	-0.91	1.29	-0.70		
48 hours post injury	-3.42	1.76	-1.94		
Cecropin A1	-1.01	1.58	-0.64		
Diptericin	-2.77	1.43	-1.94		
Drosocin	-4.72	1.73	-2.74		
Drosomycin	-5.89	1.43	-4.11		
1:1 P:C:24 hours post injury	-1.52	2.72	-0.56		
1:1 P:C:48 hours post injury	2.01	2.00	1.01		
1:1 P:C:Cecropin A1	0.10	1.08	0.09		
1:1 P:C:Diptericin	-0.99	0.87	-1.14		
1:1 P:C:Drosocin	-0.93	1.51	-0.61		
1:1 P:C:Drosomycin	1.12	0.84	1.33		
24 hours post injury:Cecropin A1	0.08	1.51	0.05		
48 hours post injury:Cecropin A1	4.90	2.53	1.94		
24 hours post injury:Diptericin	1.85	1.25	1.48		
48 hours post injury:Diptericin	5.57	1.90	2.93		
24 hours post injury:Drosocin	0.57	1.89	0.31		
48 hours post injury:Drosocin	6.41	2.88	2.23		
24 hours post injury:Drosomycin	1.18	1.30	0.91		

48 hours post injury:Drosomycin	2.98	1.82	1.64		
1:1 P:C:24 hours:Cecropin A1	3.53	3.45	1.02	7.95	0.44
1:1 P:C:48 hours:Cecropin A1	1.46	3.17	0.46		
1:1 P:C:24 hours:Diptericin	3.42	3.42	1.00		
1:1 P:C:48 hours:Diptericin	-1.61	1.99	-0.81		
1:1 P:C:24 hours:Drosocin	1.72	3.07	0.56		
1:1 P:C:48 hours:Drosocin	-3.78	3.29	-1.15		
1:1 P:C:24 hours:Drosomycin	0.87	3.41	0.25		
1:1 P:C:48 hours:Drosomycin	-2.21	1.89	-1.17		

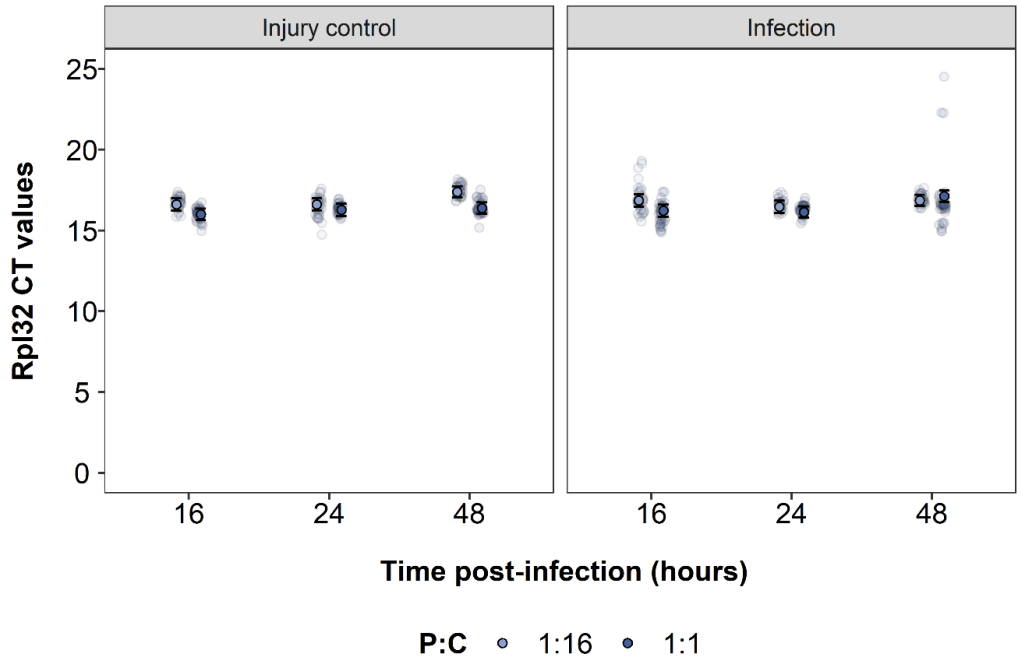

Figure S5.3: Effects of dietary P:C and time-post infection on Rpl32 reference gene C_T values for injury control and infected flies. Lighter purple points show data for the lower P:C diet (1:16 P:C) and darker purple points show data for the higher P:C diet (1:1 P:C). Linear model predictions are shown as additional data points with associated error bars showing 95% confidence intervals. Dotted line shows y = 0

Table S5.9: Summary of main effects parameter estimates and associated LRT test values for a linear mixed effects model analysing effects of diet and time after infection on the mean CT value for Rpl32 expression. The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the main effect to a model with no main effect. qPCR plate and sample ID are included as random effects. Significant results below significance level $\alpha = 0.05$ are bolded.

	Estimate	Standard error	T value	Chisq	Pr (>Chisq)
1:1 P:C	**-0.44**	**0.10**	**-4.53**	**19.80**	**<0.001**
24 hours post-treatment	**-0.05**	**0.14**	**-0.33**	**20.31**	**<0.001**
48 hours post-treatment	**0.54**	**0.15**	**3.49**		
Infection	0.07	0.09	0.81	0.69	0.41

Table S5.10: Summary of two-way interaction estimates and associated LRT test values for a linear mixed effects model analysing effects of diet and time after infection on the mean CT value for Rpl32 expression. The values are from models not including interactions with the specific main effect. Chi-squared and associated p-values are from LRT tests comparing a model with no interactions associated with the two-way interaction to a model with no two-way interaction. qPCR plate and sample ID are included as random effects. Significant results below significance level $\alpha = 0.05$ are bolded.

	Estimate	Standard error	T value	Chisq	Pr (>Chisq)
Intercept	16.70	0.19	86.81		
1:1 P:C	-0.82	0.19	-4.22		
24 hours post-treatment	0.05	0.23	0.20		
48 hours post-treatment	0.49	0.24	2.06		
Infection	0.02	0.18	0.10		
1:1 P:C:24 hours post-treatment	0.19	0.26	0.74	0.81	0.67
1:1 P:C:48 hours post-treatment	0.20	0.25	0.79		
1:1 P:C:Infection	**0.49**	**0.19**	**2.63**	**7.09**	**0.008**
24 hours post-treatment:Infection	-0.44	0.23	-1.93	3.93	0.14
48 hours post-treatment:Infection	-0.16	0.21	-0.74		

Table S5.11: Summary of a linear mixed effects model analysing effects of diet and time after infection on the mean CT value for Rpl32 expression. qPCR plate and sample ID are included as random effects. Significant results below significance level α = 0.05 are bolded.

	Estimate	Standard error	T value	Chisq	Pr (>Chisq)
Intercept	16.60	0.19	87.33		
1:1 P:C	-0.61	0.21	-2.89		
24 hours post-treatment	0.007	0.24	0.03		
48 hours post-treatment	0.79	0.24	3.22		
Infection	0.26	0.20	1.26		
1:1 P:C:24 hours post-treatment	0.28	0.34	0.81		
1:1 P:C:48 hours post-treatment	-0.40	0.31	-1.31		
1:1 P:C:Infection	-0.02	0.30	-0.08		
24 hours post-treatment:Infection	-0.40	0.32	-1.28		
48 hours post-treatment:Infection	-0.79	0.28	-2.79		
1:1 P:C:24 hours post-treatment:Infection	**0.03**	**0.44**	**0.07**	**13.50**	**0.001**
1:1 P:C:48 hours post-treatment:Infection	**1.31**	**0.41**	**3.21**		